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Congenital Disorders of Glycosylation

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Abbreviations

9-ADM	9-anthryldiazomethane
ALG	asparagine-linked glycosylation
CDG	Congenital Disorders of Glycosylation
CDT	carbohydrate-deficient transferrin
CTP	cytidine triphosphate
EGFP	enhanced green fluorescent protein
ER	endoplasmic reticulum
ESI-MS	electrospray ionization mass spectrometry
DNA	desoxyribonucleic acid
DPM	dolichol-phosphate mannosyltransferase
Dol	dolichol
Dol-P	dolichol-phosphate
FCS	fetal calf serum
GDP	guanosine diphosphate
Glc	glucose
GlcNAc	N-acetylglucosamine
GPI	glycosylphosphatidylinositol
HMG-CoA	3-hydroxy-3-methyl-glutaryl-Coenzyme A
HPLC	high performance liquid chromatography
IEF	isoelectric focusing
LLO	lipid-linked oligosaccharide
Man	mannose
MPI	mannose phosphate isomerase
MRI	magnetic resonance imaging
NLO	N-linked oligosaccharide
PCR	polymerase chain reaction
PLE	protein-losing enteropathy
OMIM	Online Mendelian Inheritance in Man
OST	oligosaccharyltransferase
PMM	phosphomannomutase
RNA	ribonucleic acid
TM	transmembrane
UDP	uridine diphosphate
ZA	zaragozic acid A

Summary

Protein N-glycosylation is an essential posttranslational modification, which diversifies nearly half of the human proteins. The attached glycans contribute to folding and targeting of the glycoproteins within eukaryotic cells, and after secretion to the extracellular space they account for cell-cell interactions and signalling. Unique in N- glycosylation is the formation of a lipid-linked oligosaccharide (LLO) precursor, which is transferred *en bloc* to selected asparagine (N)-residues during protein translation. The biosynthesis of the precursor $GlcNAc_2Man_9Glc_3$ takes place on the lipid anchor dolichol-phosphate (Dol-P) in the endoplasmic reticulum (ER). The first steps leading to the formation of the Dol-PP-$GlcNAc_2Man_5$ intermediate are catalysed at the cytoplasmatic side of the ER. After translocation of this intermediate across the membrane, the LLO is completed at the luminal side. Trimming and further modifications of N-linked glycans occur in the ER and after translocation to the Golgi apparatus.

Mutations in genes encoding glycosyltransferases and other proteins involved in either the biosynthesis of the LLO precursor or the modification and transport of N-glycoproteins lead to inherited human diseases called Congenital Disorders of Glycosylation (CDG). Patients show a broad range of multi-systemic symptoms, which impair predominately the nervous system. Around 20% of the CDG patients die in early childhood. CDG are commonly diagnosed by isoelectric focusing (IEF) of the serum glycoprotein transferrin, which allows the classification into the subgroups of biosynthetic and processing defects.

At the beginning of this thesis, twelve biosynthetic N-glycosylation defects had been characterized and only one subtype, namely mannose phosphate isomerase (MPI) deficiency, could be effectively treated by oral Man supplementation. Accordingly, the aims of this work were to identify the molecular basis of untyped CDG cases. In addition, a sensitive method for the analysis of Dol-P should be established to screen for defects affecting the biosynthesis of the lipid carrier in the ER. Considering the common biosynthetic pathway of Dol and cholesterol, various cholesterol lowering drugs should be tested in a therapeutical approach for their ability to enhance N-glycosylation.

In the first part of the thesis, we could demonstrate that dysfunction of the ER membrane protein RFT1 leads to the accumulation of the LLO intermediate $GlcNAc_2Man_5$ in an untyped CDG patient. This feature together with the detection of complete N-glycans on proteins suggested a disturbed translocation of the Dol-linked heptasaccharide across the ER membrane. Complementation assays using an Rft1 deficient yeast strain showed that the identified mutation was indeed pathogenic. This original description of RFT1 deficiency as a case of CDG led to the rapid identification of additional cases. In the next study, the detailed examination of three supplemental patients further established the definition of the clinical scope of the novel

disease. Furthermore, the molecular analysis of another case of ALG8 glucosyltransferase deficiency also contributed to a better definition of the genotype : phenotype relationship.

Dol is essential for proper N-glycosylation. So far, only a deficiency of the Dol-kinase has been identified as cause of CDG. This isolated case is partially due to the lack of sensitive methods enabling the detection of Dol defects. We addressed this task in a combined approach using both fluorescent derivatization followed by HPLC and direct electrospray ionization mass spectrometry (ESI-MS). This new method now allows the determination of the polyisoprene chain length of compound Dol-P, the assignment of the saturation state of the α-subunit and the quantification of distinct Dol-P in cultured cells.

The majority of the CDG mutations only diminish the catalytic activity of the affected enzymes. Enhanced substrate availability, such as of the membrane anchor Dol-P, might therefore stimulate reduced N-glycosylation observed in CDG patient fibroblasts. Accordingly, we could show that modulation of the cholesterol biosynthetic pathway by the squalene synthase inhibitor zaragozic acid A (ZA) leads to increased formation of Dol-P. As expected, the enhanced availability of Dol-P yielded in elevated levels of the monosaccharide donor Dol-P-Man in Dol-P-Man synthase subunit 1 (DPM1) deficient fibroblasts. Though, not only the formation of this sugar building block is stimulated, but also pathogenic accumulations and alterations of LLO and N-linked glycans could be corrected upon ZA administration. These findings indicate the potential of ZA as therapeutical agent for CDG.

In conclusion, the characterization of a novel CDG subtype improved the comprehension of the biological and clinical importance of the RFT1 protein. The establishment of a sensitive Dol-P analysis method and the promising effect of ZA on N-glycosylation in CDG contributed to set a new state of the art in the field of CDG research.

Zusammenfassung

Protein N-Glykosylierung ist eine essentielle posttranslationale Modifikation, welche nahezu die Hälfte aller menschlichen Proteine diversifiziert. Die angehängten Zuckermoleküle gewährleisten die Faltung sowie die zellinterne Verteilung der Glycoproteine und tragen im extrazellulären Raum zu interzellulären Interaktionen bei. Einzigartig bei der N-Glykosylierung ist die Bildung eines Lipid gebundenen Mehrfachzuckers (LLO), welcher während der Proteintranslation als Ganzes auf ausgewählte Asparagine (N) übertragen wird. Die Biosynthese des $GlcNAc_2Man_9Glc_3$ Vorläufers wird durch den Lipidanker Dolichol-phosphat (Dol-P) an die Membran des Endoplasmatischen Retikulums (ER) gebunden. Die ersten Schritte, welche zur Bildung des Zwischenproduktes Dol-PP-$GlcNAc_2Man_5$ führen, werden auf der zytoplasmatischen Seite des ER katalysiert. Nach dem Passieren der Membran wird der Vorläufer im Lumen vervollständigt. Das Trimmen sowie weitere Modifikationen der Protein gebundenen Zuckermoleküle geschehen im ER oder nach dem Weitertransport zum Golgi Apparat.

Mutationen in Genen, welche für Glykosyltransferasen oder andere Proteine kodieren, die entweder in die Biosynthese des LLO Vorläufers oder in die Modifikationen und den Transport von N-Glykoproteinen involviert sind, führen zu menschlichen Erbkrankheiten, welche Kongenitale Glykosylierungsstörungen (CDG) genannt werden. Die Patienten weisen eine Vielzahl von Symptomen auf, welche zwar diverse Organe betreffen können, aber hauptsächlich das Nervensystem beeinträchtigen. Bei ungefähr 20% der Patienten ist die Krankheit so schwerwiegend, dass sie noch in früher Kindheit versterben. CDG wird gewöhnlich mittels isoelektrischer Fokussierung (IEF) des Serumproteins Transferrin diagnostiziert und diese Methode erlaubt gleichzeitig die Einteilung in Biosynthese- und Prozessierungsdefekte.

Zu Beginn der Doktorarbeit waren zwölf Biosynthesedefekte charakterisiert gewesen und nur einer davon, nämlich die Mannose-phosphat Isomerase (MPI) Defizienz, konnte mittels oraler Mannose Verabreichung behandelt werden. Dementsprechend wurde das Verknüpfen von nicht zugeordneten CDG Patienten mit den zugrunde liegenden Gendefekten und die damit einhergehende Identifikation von neuen Subtypen als eines der Ziele definiert. Zudem sollte eine sensitive Methode entwickelt werden, mit welcher Dol-P analysiert werden können um so nach Defekten in der Biosynthese des Lipides zu suchen. In Anbetracht der gemeinsamen Biosynthese von Dol und Cholesterin sollte schlussendlich in einem therapeutischen Versuch die Fähigkeit von Cholesterin senkenden Mittel zur Steigerung der N-Glykosylierung untersucht werden.

Im ersten Teil dieser Arbeit konnten wir zeigen, dass eine Fehlfunktion des ER Membranproteins RFT1 die Ansammlung des LLO $GlcNAc_2Man_5$ in einer CDG Patientin verursacht. Zusammen mit dem Nachweis von vollständigen Protein gebundenen Zuckermolekülen führte dies zur Vermutung, dass der Transport des Siebenfachzuckers durch

die ER Membran gestört sein könnte. Mit Hilfe eines Komplementierungsassays in einem Rft1 defekten Hefestamm konnte gezeigt werden, dass die identifizierte Mutation wirklich pathogen ist. In der folgenden Studie ermöglichte die detaillierte Untersuchung von drei zusätzlichen Patienten die Definition des klinischen Phänotyps von RFT1-CDG. Des Weiteren trugen molekularen Analysen dazu bei, dass ein fünfter Fall von ALG8 Glukosyltransferase Defizienz identifiziert und damit das Verhältnis zwischen Genotyp und Phänotyp besser definiert werden konnte.

Dol ist unabdingbar für eine einwandfreie Funktion der N-Glykosylierung. Bis jetzt wurde nur ein Defekt der Dol-Kinase als Ursache von CDG identifiziert. Die bisherige Einzigartigkeit dieses Falls kann teilweise durch das Fehlen einer sensitiven Methode erklärt werden, welche Dol Defekte detektieren könnte. Wir lösten dieses Manko in einem kombinierten Ansatz unter Verwendung von Fluoreszenzmarkierung und anschliessender HPLC sowie mittels direkter Elektrospray Ionisierung Massenspektrometrie (ESI-MS). Anhand dieser neuen Methode konnten die exakten Polyisoprenlängen einer Dol-P Mischung bestimmt werden, zusätzlich der Sättigungszustand der α-Untereinheit ermittelt sowie die genauen Mengen von verschiedenen Dol-P in Zellextrakten gemessen werden.

Die Mehrheit der CDG Mutationen vermindert die katalytische Aktivität der betroffenen Enzyme nur zu einem gewissen Grad. Eine vermehrte Substratverfügbarkeit, zum Beispiel des Membranankers Dol-P, könnte daher die reduzierte N-Glykosylierung in CDG Patienten Fibroblasten stimulieren. Eine Modulation der Cholesterin Biosynthese durch den Squalensynthase Hemmstoff Zaragozic Acid A (ZA) führte nachweislich zur vermehrten Bildung von Dol-P. Wie erhofft hatte die erhöhte Verfügbarkeit von Dol-P zur Folge, dass auch der Level von Dol-P-Man in Kontrollzellen und Dol-P-Man Synthase Untereinheit 1 (DPM1) defekten Fibroblasten anstieg. Aber nicht nur die Bildung dieses Zuckerbausteins konnte angeregt werden, sondern auch krankhaften Ansammlungen und Veränderungen von LLO sowie von Protein gebunden Zuckermolekülen konnten durch die Verabreichung von ZA verbessert werden. Diese vielversprechenden Resultate deuten das Potential von ZA als mögliches CDG Therapiemittel an.

Zusammengefasst lässt sich sagen, dass die Charakterisierung eines neuen CDG Subtyps viel zum Verständnis der biologischen und klinischen Bedeutung des RFT1 Proteins beigetragen hat. Durch die Etablierung einer Dol-P Analysemethode and der überaus positive Effekt von ZA auf die N-Glykosylierung in CDG tragen dazu bei, dass die CDG Forschung ein beachtliches Stück vorangetrieben werden konnte.

Introduction

Congenital Disorders of Glycosylation: an Update on Defects Affecting the Biosynthesis of Dolichol-linked Oligosaccharides

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Congenital Disorders of Glycosylation: an Update on Defects Affecting the Biosynthesis of Dolichol-Linked Oligosaccharides

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Abstract

Defects in the biosynthesis of the oligosaccharide precursor for N-glycosylation lead to decreased occupancy of glycosylation sites and thereby to diseases known as Congenital Disorders of Glycosylation (CDG). In the last 20 years, approximately 1000 CDG patients have been identified presenting with multiple organ dysfunctions. This review sets the state of the art by listing all mutations identified in the 15 genes (*PMM2*, *MPI*, *DPAGT1*, *ALG1*, *ALG2*, *ALG3*, *ALG9*, *ALG12*, *ALG6*, *ALG8*, *DOLK*, *DPM1*, *DPM3*, *MPDU1* and *RFT1*) yielding a deficiency of dolichol-linked oligosaccharide biosynthesis. The present analysis shows that most mutations lead to substitutions of strongly conserved amino acid residues across eukaryotes. Furthermore, the comparison between the different forms of CDG affecting dolichol-linked oligosaccharide biosynthesis shows that the severity of the disease does not relate to the position of the mutated gene along this biosynthetic pathway.

Key Words

Glycosylation, endoplasmic reticulum, CDG, dolichol, glycoprotein

Abbreviations

ALG, asparagine-linked glycosylation; CDG, Congenital Disorders of Glycosylation; ER, endoplasmic reticulum; IEF, isoelectric focusing; OST, oligosaccharyltransferase; MPI, mannose phosphate isomerase; PMM, phosphomannomutase; TM, transmembrane

Introduction

N-glycosylation is an essential form of post-translational modification in eukaryotes. Several types of N-glycosylation disorders have been described over the last decade, thereby expanding the list of congenital disorders of glycosylation (CDG) (Freeze, 2006). Looking at the N-glycosylation disorders identified so far, it is reasonable to predict that all genes involved in the biosynthesis of N-glycans are likely to be once associated with a form of CDG. Despite the achievement of the last years, the identification of novel N-glycosylation disorders remains challenging, mainly because of their rarity and because of their rather nonspecific clinical pictures (Leroy, 2006). The widespread application of a simple isoelectric focusing (IEF) test allowing the detection of underglycosylated serum transferrin (van Eijk, et al., 1983) has been instrumental in pointing at potential cases of N-glycosylation disorders. The pattern of transferrin glycoforms obtained by IEF usually allows differentiating between defects of N-glycosylation site occupancy and defects of N-glycan trimming and elongation. These two groups of defects have been originally defined as CDG type-I or CDG-I, and CDG type-II or CDG-II, respectively (Aebi, et al., 1999). Defects in other classes of glycosylation and in sugar-nucleotide transporters have also been designated as CDG-II (Lübke, et al., 2001; Topaz, et al., 2004; Martinez-Duncker, et al., 2005). However, the recent description of glycosylation disorders caused by defects of vesicular transport (Wu, et al., 2004; Foulquier, et al., 2006; Foulquier, et al., 2007), which by definition fall in the category of CDG-II, has prompted for a revision of the CDG nomenclature. Along this line, it has been suggested to abandon the differentiation between CDG-I and -II, and to name the glycosylation disorders by using the official abbreviation of the defective gene (Jaeken, et al., 2008).

Since the first description of mutations in the phosphomannomutase-2 (*PMM2*) gene as causing CDG (Matthijs, et al., 1997), 25 additional disorders of N-glycosylation have been identified (Jaeken, et al., 2008). Clinically, most of these disorders lead to psychomotor retardation with variable neuromuscular involvement and additional features like hormonal abnormalities and coagulopathies (Leroy, 2006). The severity of these symptoms often varies tremendously, ranging from slight mental retardation to multiorgan dysfunctions often associated to infantile lethality. A typical question arising when comparing the clinical manifestations described is the following: does the severity of a CDG case relate to the position of the defect along the N-glycosylation biosynthetic pathway or rather to the degree of inactivation conferred by the mutation underlying the gene defect? This question can be particularly well examined when considering the glycosylation disorders caused by defective assembly of the dolichol-linked oligosaccharides since this biosynthetic pathway is sequential (Figure 1). Moreover, the assembly of dolichol-linked oligosaccharides is strongly conserved among eukaryotes, thereby enabling the application of asparagine-linked glycosylation (ALG) mutant strains of the yeast

Saccharomyces cerevisiae as tools for investigating the functional impact of human mutations (Westphal, et al., 2001a; Grubenmann, et al., 2004; Haeuptle, et al., 2008).

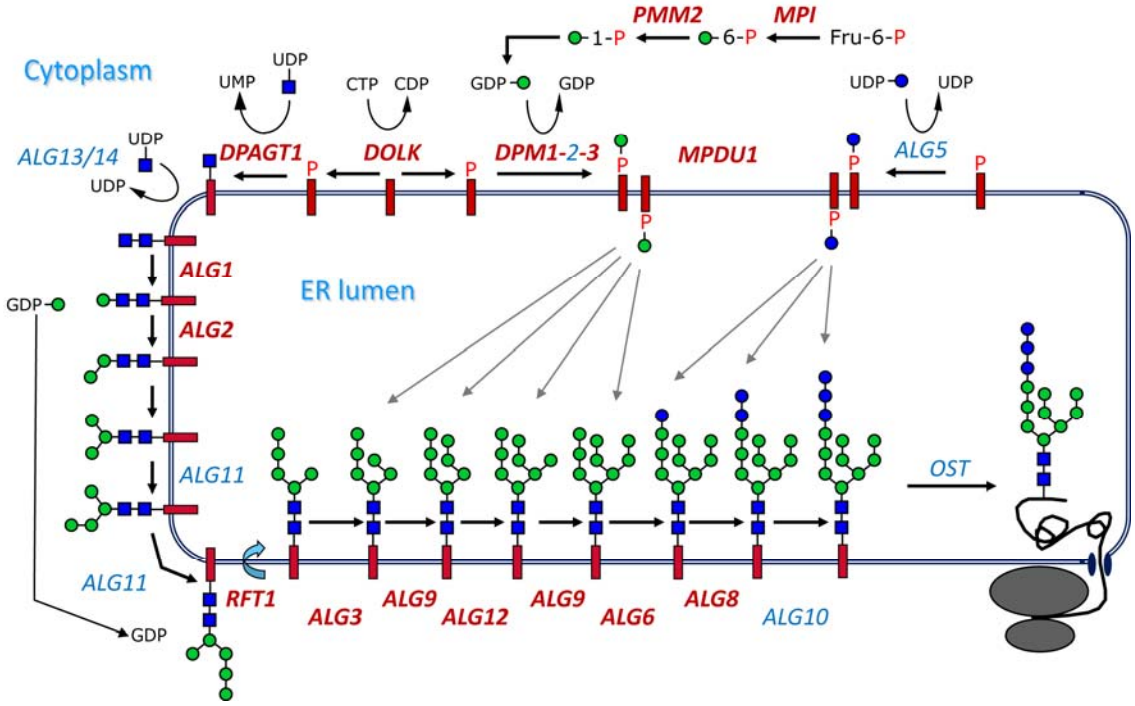

Figure 1. Pathway of dolichol-linked oligosaccharide biosynthesis. After phosphorylation (P) of the lipid carrier dolichol (red bar), two GlcNAc (blue box), nine Man (green circle) and three Glc (blue circle) units are successively added by various glycosyltransferases. Nucleotide activated monosaccharides serve as donor substrates for the cytosolically oriented enzymes. After being translocated into the endoplasmic reticulum (ER) lumen, the intermediate dolichol-PP-$GlcNAc_2Man_5$ is further extended by luminally acting mannosyl- and glucosyltransferases using dolichol-P activated Man and Glc as sugar building blocks. The complete structure dolichol-PP-$GlcNAc_2Man_9Glc_3$ is transferred to selected asparagines on newly synthesized glycoproteins by the oligosaccharyltransferase (OST) complex. The gene symbols are indicated next to the catalyzed reactions. The 15 genes associated with CDG are marked in red.

The aim of this review is to define the state of the art for the known disorders of dolichol-linked oligosaccharide biosynthesis and especially to discuss the effect of identified mutations on the functions of the affected proteins. To date, 15 gene defects have been described in this group (Matthijs, et al., 1997; Niehues, et al., 1998; Imbach, et al., 1999; Körner, et al., 1999; Imbach, et al., 2000b; Schenk, et al., 2001b; Chantret, et al., 2002; Chantret, et al., 2003; Thiel, et al., 2003; Wu, et al., 2003; Frank, et al., 2004; Grubenmann, et al., 2004; Kranz, et al., 2007b; Haeuptle, et al., 2008; Lefeber, et al., 2009). Common for all of these defects is the insufficient supply of dolichol-linked oligosaccharide precursor, which leads to decreased occupancy of N-glycosylation sites.

Table 1. Gene defects leading to deficient assembly of dolichol-linked oligosaccharides

Gene	OMIM [a]	Enzyme	Disorder [b]	Disorder [c]	OMIM [a]	Mutations	Patients
PMM2	601785	Phosphomannomutase 2	CDG-Ia	PMM2-CDG	212065	103	> 800
MPI	154550	Mannose phosphate isomerase	CDG-Ib	MPI-CDG	602579	18	>25
DPAGT1	191350	GlcNAc-1-P transferase	CDG-Ij	DPAGT1-CDG	608093	3	3
ALG1	605907	Mannosyltransferase 1	CDG-Ik	ALG1-CDG	608540	4	7
ALG2	607905	Mannosyltransferase 2	CDG-Ii	ALG2-CDG	607906	2	1
ALG3	608750	Mannosyltransferase 6	CDG-Id	ALG3-CDG	601110	9	11
ALG9	606941	Mannosyltransferase 7-9	CDG-Il	ALG9-CDG	608776	2	3
ALG12	607144	Mannosyltransferase 8	CDG-Ig	ALG12-CDG	607143	11	8
ALG6	604566	Glucosyltransferase 1	CDG-Ic	ALG6-CDG	603147	20	>36
ALG8	608103	Glucosyltransferase 2	CDG-Ih	ALG8-CDG	608104	12	9
DOLK	610746	Dolichol kinase	CDG-Im	DOLK-CDG	610768	2	4
DPM1	603503	Dolichol-P mannosyltransferase 1	CDG-Ie	DPM1-CDG	608799	6	8
DPM3	605951	Dolichol-P mannosyltransferase 3	CDG-Io	DPM3-CDG	612937	1	1
MPDU1	604041	Man-P-dolichol utilization defect 1	CDG-If	MPDU1-CDG	609180	5	5
RFT1	611908	RFT1 homolog (*S. cerevisiae*)	CDG-In	RFT1-CDG	612015	5	6

[a] http://www.ncbi.nlm.nih.gov/sites/entrez?db=OMIM

[b] According to the recommended nomenclature of 1999 (Aebi, et al., 1999)

[c] According to the recommended nomenclature of 2008 (Jaeken, et al., 2008)

The defective glycosylation reactions can be organized in five categories based on enzymatic activity and on subcellular localization (Fig. 1). The first category comprises the cytosolic enzymes PMM2 and mannose phosphate isomerase (MPI) (Matthijs, et al., 1997; Niehues, et al.,

1998). The second category includes the N-acetylglucosaminyl- and mannosyltransferase enzymes involved in the assembly of the dolichol-linked oligosaccharide at the cytosolic side of the endoplasmic reticulum (ER) membrane (Thiel, et al., 2003; Wu, et al., 2003; Grubenmann, et al., 2004). The third and forth categories include the mannosyltransferase and glucosyltransferase enzymes that elongate the luminally-oriented dolichol-linked oligosaccharide (Imbach, et al., 1999; Körner, et al., 1999; Chantret, et al., 2002; Chantret, et al., 2003; Frank, et al., 2004), respectively, whereas the last category comprises the proteins that modify dolichol or affect the availability of dolichol-linked carbohydrates to the assembly pathway (Imbach, et al., 2000b; Schenk, et al., 2001b; Kranz, et al., 2007b; Haeuptle, et al., 2008; Lefeber, et al., 2009).

Over the last decade, close to 1000 CDG patients were diagnosed with a disorder of dolichol-linked oligosaccharide assembly, thereby unravelling more than 200 mutations in 15 genes (Table 1). The present review provides a comprehensive overview of the pathway by mapping these mutations on model representations of the affected proteins. By integrating the clinical features associated with each mutation, this overview enables a discussion of the importance of individual proteins in the context of N-glycosylation output and human physiology.

Group 1: Cytosolic enzymes

The glycosyltransferase enzymes of the N-glycosylation pathway use nucleotide- or dolichol-activated monosaccharides as donor substrates. Defects in the biosynthesis of these substrates lead to CDG. The formation of GDP-Man occupies a central stage in the process considering the nine Man residues constituting the N-glycan core (Fig. 1). Accordingly, the cytosolic enzymes PMM2 and MPI, which catalyze the conversion of Man-6-P to Man-1-P and of fructose-6-P to Man-6-P, respectively, are essential members of the N-glycosylation pathway. Note worthily, PMM2 and MPI deficiencies affect additional glycosylation pathways, such as O-mannosylation and glycosylphosphatidylinositol-anchor biosynthesis, which also rely on Man-based donor substrates.

PMM2 (PMM2-CDG, CDG-Ia)

More than 800 patients have been identified with mutations in the *PMM2* gene (PMM2-CDG), thereby constituting the largest group of CDG cases (Table 1). Clinically, neurologic symptoms including psychomotor retardation, developmental delay, epilepsy, ataxia, cerebellar hypoplasia and visual impairment are predominant. Furthermore, symptoms of coagulopathy, hypotonia, cardiomyopathy, gastrointestinal and hepatic problems are also frequently observed. Strong dysmorphic features including severe skeletal deformities are found in most cases (Mizugishi, et al., 1999; Westphal, et al., 2001a; Briones, et al., 2002; Tayebi, et al., 2002; Ono, et al., 2003; Coman, et al., 2005; Schollen, et al., 2007; Vermeer, et al., 2007; Wurm, et al., 2007; Truin, et al., 2008; Perez-Duenas, et al., 2009; Thong, et al., 2009; Vega, et al., 2009). A mortality rate of over 20% within the first years of life is frequent in cases presenting with low residual PMM2 activity (Matthijs, et al., 2000). The essential role of PMM2 is supported by finding that disruption of this gene in mice leads to early embryonic lethality (Thiel, et al., 2006).

PMM2-CDG is the most frequent form of CDG in respect to the number of mutations identified to date, which sums up to 103 (Supp. Table S1) (Matthijs, et al., 1997; Matthijs, et al., 1999; Mizugishi, et al., 1999; Matthijs, et al., 2000; Grunewald, et al., 2001; Westphal, et al., 2001a; Briones, et al., 2002; Schollen, et al., 2002; Tayebi, et al., 2002; Callewaert, et al., 2003; Ono, et al., 2003; Coman, et al., 2005; Le Bizec, et al., 2005; Vuillaumier-Barrot, et al., 2006; Schollen, et al., 2007; Vermeer, et al., 2007; Wurm, et al., 2007; Truin, et al., 2008; Perez-Duenas, et al., 2009; Thong, et al., 2009; Vega, et al., 2009). Three of them (p.Q37H, p.E197A and p.A233T) occur only in combination with two other heterozygous mutations and are therefore assumed to be single nucleotide polymorphisms. The mutations are scattered over the *PMM2* gene and yield a broad range of protein defects (Fig. 2; Supp. Table S1). A total of 80 missense mutations affect 68 different amino acid residues. Five nonsense mutations introduce an early stop codon and lead to truncated PMM2 proteins. The seven base deletion and insertion mutations identified to date

lead to frame shifts and thus to truncated proteins. A total of ten splicing defects have been described, whereas the causative mutations were not only found at the splicing sites, but also as far as 15 kb in intronic regions (Schollen, et al., 2007; Truin, et al., 2008; Vega, et al., 2009). Finally, one patient displayed the complete loss of exon 8 due to a deletion mediated by an Alu retro-transposition (Schollen, et al., 2007).

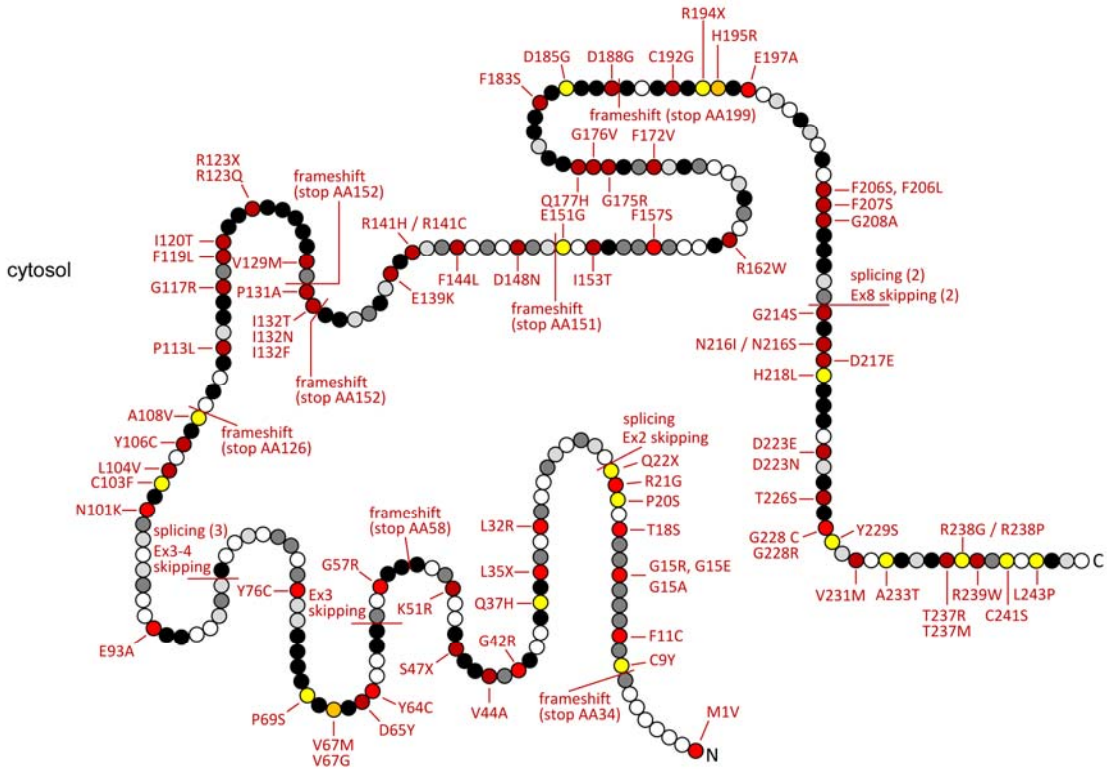

Figure 2. Schematic model of PMM2. PMM2 protein sequences were aligned using the ClustalW program (Thompson, et al., 1994). The *Homo sapiens* sequence (NP_000294.1) was compared to the rodent *Mus musculus* (NP_058577.1), to the zebra fish *Danio rerio* (NP_956378.1), to the fruit fly *Drosophila melanogaster* (NP_648589.1), to the nematode *Caenorhabditis elegans* (NP_502698.2) and to the budding yeast *Saccharomyces cerevisiae* (NP_116609.1). Sequences were obtained from the Genbank (http://www.ncbi.nlm.nih.gov/ Genbank/index.html). Black dots represent strictly conserved amino acids and dark gray, light gray, white, those amino acids with a conservation of 83%, 67% and less than 67%, respectively. Mis- and nonsense mutations are marked next to the affect amino acids. The dark red, light red, orange and yellow dots mark the mutated amino acids that are conserved at 100%, 83%, 67% and less than 67%, respectively. Splicing defects are marked with a bar and explicitly entitled in the case of assigned exon skipping. Further or unknown implications on the protein level of the affected enzyme are termed with splicing. Deletion or insertion mutations causing a frame-shift are marked with a bar as well and entitled as frame-shift, whereas the position of the resulting premature stop codon is given in brackets.

The human PMM2 enzyme is 246 amino acids long and shows a very high level of amino acid conservation among eukaryotes (Fig. 2). The crystal structure of its isozyme PMM1 has been solved (Silvaggi, et al., 2006), which by extrapolation contributes to assign the impact of mutations on PMM2 protein function and stability. Interestingly, none of the amino acids constituting the active center have been found to be mutated in CDG. However, several mutations have been identified leading to declined substrate or cofactor binding. The arginines at position p.R150, p.R123 and p.R28 in PMM1 (equivalent to p.R141, p.R123 and p.R21 in PMM2) were shown to be involved in binding either the phosphate or the C2 hydroxyl group of the substrate (Silvaggi, et al., 2006). Substitution of these amino acids in PMM2 (Fig. 2) results in a severe clinical phenotype and it has been shown that homozygosity for the p.R141H mutation is not compatible with life (Matthijs, et al., 1998). The homodimeric interaction of PMM1 is mediated by a hydrophobic core, supported by surrounding hydrogen bonds and salt bridges (Silvaggi, et al., 2006). The mutations p.L104V, p.F119L, p.I120T in PMM2 might disrupt the analogous hydrophobic core (p.L113, p.F128 and p.I129 in PMM1). Furthermore, the mutations p.E93A and p.N101K (p.E102 and p.N110 in PMM1) are suspected to impair the homodimeric interaction by deletion of a hydrogen bond and a salt bridge (Fig. 2) (Silvaggi, et al., 2006). Other mutations have been shown to destabilize the overall protein fold and thereby leading to reduced catalytic activity (Pirard, et al., 1999).

MPI (MPI-CDG, CDG-Ib)

MPI-CDG can be detected biochemically by measuring MPI activity in patients' fibroblasts or leukocytes (Jaeken, et al., 1998). In doing so, 25 patients have been identified so far, exhibiting 18 different mutations in the *MPI* gene (Jaeken, et al., 1998; Niehues, et al., 1998; Babovic-Vuksanovic, et al., 1999; de Lonlay, et al., 1999; Schollen, et al., 2000a; Westphal, et al., 2001b; Schollen, et al., 2002; Vuillaumier-Barrot, et al., 2002; Penel-Capelle, et al., 2003; Vuillaumier-Barrot, 2005). The clinical presentation of these patients is unique due to the fact that neurological symptoms are usually absent. Mostly affected are the gastrointestinal tract and the liver with symptoms like diarrhoea, vomiting, gastrointestinal bleeding, protein-losing enteropathy, hepatomegaly and hepatic fibrosis. Additionally, coagulopathy, hypoglycaemia and thrombotic events have been observed in moderate to severe cases including at least six lethal outcomes (Jaeken, et al., 1998; Niehues, et al., 1998; Babovic-Vuksanovic, et al., 1999; de Lonlay, et al., 1999; Westphal, et al., 2001b; Vuillaumier-Barrot, et al., 2002; Penel-Capelle, et al., 2003). Interestingly, MPI-CDG can effectively be treated by Man supplementation (Niehues, et al., 1998; Babovic-Vuksanovic, et al., 1999; de Lonlay, et al., 1999; Westphal, et al., 2001b; Penel-Capelle, et al., 2003). The orally applied Man could be phosphorylated by hexokinases yielding Man-6-P,

and thereby enabling the functional bypass of the defective isomerase step (Panneerselvam and Freeze, 1996).

The human MPI enzyme is a soluble cytosolic protein of 423 amino acids (Fig. 3). The crystal structure of *Candida albicans* MPI (Cleasby, et al., 1996) suggests that the protein is a metallo-enzyme containing one zinc atom. Whereas most MPI domains show a rather low conservation status among eukaryotes, the central domain, forming the catalytic cleft, is highly conserved (Cleasby, et al., 1996). The human MPI shares 42% sequence homology with its *C. albicans* ortholog and is therefore assumed to present a similar protein fold.

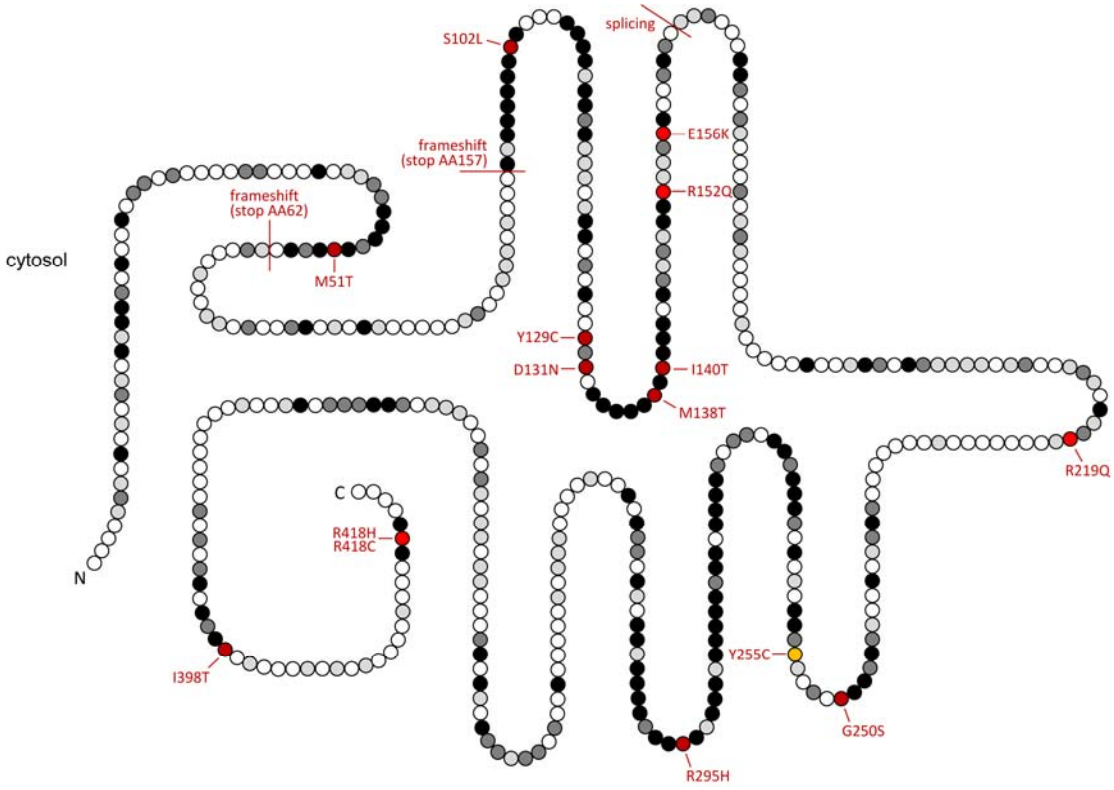

Figure 3. Schematic model of MPI. Mutations and the conservation of the MPI amino acids among the human (NP_002426.1), mouse (NP_080113.1), zebra fish (NP_001028282.1), fruit fly (NP_649940.1), nematode (NP_499174.3) and budding yeast (NP_010918.1) proteins were mapped as in Figure 2.

The 18 known mutations (Supp. Table S2) comprise a group of 15 point mutations, while only two frame-shift causing mutations and one splicing defect have been reported. Seven of these missense mutations map to the predicted catalytic domain, which ranges from cysteine p.C11 to phenylalanine p.F151 and from leucine p.L257 to arginine p.R322 based on the *C. albicans* MPI crystal structure (Cleasby, et al., 1996) (Fig. 3). The mutations of strictly conserved residues, such as p.M51T, p.S102L, p.M138T and p.I140T, lie in closest proximity to the active site and

might directly impede the enzymatic activity. The p.R295H mutation converts a strictly conserved arginine, which corresponds to p.R304 in the *C. albicans* enzyme. This positively charged residue is positioned at the border of the active site cleft and is proposed to be responsible for substrate phosphate binding (Cleasby, et al., 1996). The remaining missense mutations alter amino acids of various degrees of conservation, thereby leading most probably to a destabilization of the MPI protein fold (Fig. 3).

Group 2: Cytosolically acting glycosyltransferases

The assembly of the dolichol-linked oligosaccharide required for N-glycosylation is initiated on the cytosolic side of the ER membrane and proceeds up to the formation of the intermediate dolichol-PP-$GlcNAc_2Man_5$ (Fig. 1). Mutations in genes encoding three involved glycosyl-transferases, namely *DPAGT1*, *ALG1* and *ALG2*, have been found to cause CDG.

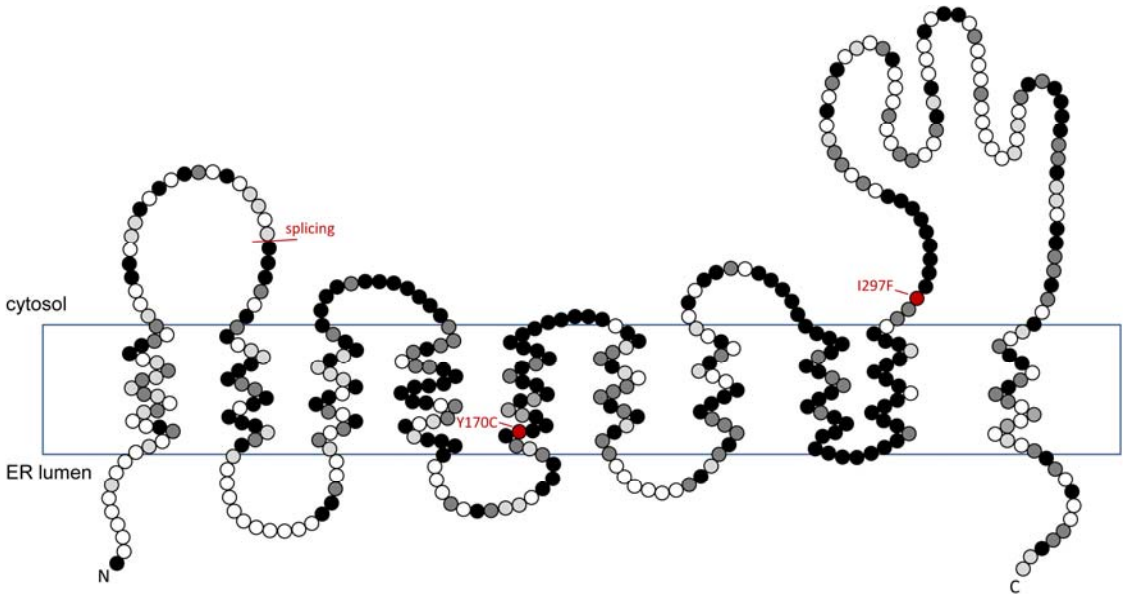

Figure 4. Schematic model of DPAGT1. Mutations and the conservation of the DPAGT1 amino acids among the human (NP_001373.2), mouse (NP_031901.2), zebra fish (NP_001082880.1), fruit fly (NP_609608.1), nematode (NP_507859.2) and budding yeast (NP_009802.1) proteins were mapped as in Figure 2. The membrane topology prediction of the DPAGT1 enzyme was performed running the TMpred (http://www.ch.embnet.org/software/TMPRED_form.html), the TMHMM (http://www.cbs.dtu.dk/services/TMHMM-2.0/) (Krogh, et al., 2001), the DAS (http://www.sbc.su.se/~miklos/DAS/) (Cserzo, et al., 1997) and the SOSUI (http://bp.nuap.nagoya-u.ac.jp/sosui/sosui_submit.html) (Hirokawa, et al., 1998) algorithms.

DPAGT1 (DPAGT1-CDG, CDG-Ij)

Deficiency of the UDP-GlcNAc:dolichol-phosphate GlcNAc-1-P transferase initiating the biosynthetic pathway leads to DPAGT1-CDG (Wu, et al., 2003). With only three patients and three mutations identified, it represents a rare form of CDG (Wu, et al., 2003; Vuillaumier-Barrot, 2005). The clinical manifestations associated to DPAGT1 deficiency are unclear since only one of the three patients has been described clinically. This case presented with a developmental delay, microcephaly and exotropia, mental retardation, severe hypotonia and intractable seizures (Wu, et al., 2003). Unfortunately, the clinical description of the two other DPAGT1-CDG patients, a pair of siblings, is not available (Vuillaumier-Barrot, 2005).

The human DPAGT1 is a hydrophobic protein of 408 amino acids that is predicted to span the ER membrane ten times (Fig. 4). The five loops assumed to protrude to the cytosol are mostly conserved among eukaryotes, which supports their function as part of the active center. One of the two identified missense mutations alters a highly conserved isoleucine at position p.I297, which localizes to the last of the five cytosolic loops (Fig. 4; Supp. Table S3). The other point mutation (p.Y170C), which maps to the fifth transmembrane (TM) domain, was found in combination with a splicing defect originating from an unknown genetic reason (Wu, et al., 2003). The third identified mutation is another splicing defect, assigned to a mutation within the first intron (c.162-8G>A) (Vuillaumier-Barrot, 2005).

Mannosyltransferase 1 (ALG1-CDG, CDG-Ik)

The phenotype of ALG1-CDG is very severe, given that at least four of seven patients died in childhood. Common symptoms are dysmorphic features, microcephaly, intractable seizures, hypotonia, coagulopathy and visual impairment (de Koning, et al., 1998; Grubenmann, et al., 2004; Kranz, et al., 2004; Schwarz, et al., 2004). Individual patients present with nearly the complete set of CDG symptoms including immunoglobulin G deficiency (Kranz, et al., 2004) and recurrent non-immune hydrops fetalis (de Koning, et al., 1998).

The human *ALG1* gene encodes a β-1,4 mannosyltransferase, which catalyses the addition of the first Man to dolichol-PP-linked chitobiose. The 464 amino acid protein is predicted to have a type-I ER membrane topology with a large cytosolic C-terminal domain harbouring the active site (Fig. 5). To date, four *ALG1* mutations have been described (Supp. Table S4) (de Koning, et al., 1998; Grubenmann, et al., 2004; Kranz, et al., 2004; Schwarz, et al., 2004). However, one of three heterozygous point mutations (p.D429E) mapped in a single CDG patient, most likely represents a single nucleotide polymorphism (Grubenmann, et al., 2004). The residual three point mutations are spread all over the cytosolic domain, where they mainly convert weakly conserved amino acids (Fig. 5).

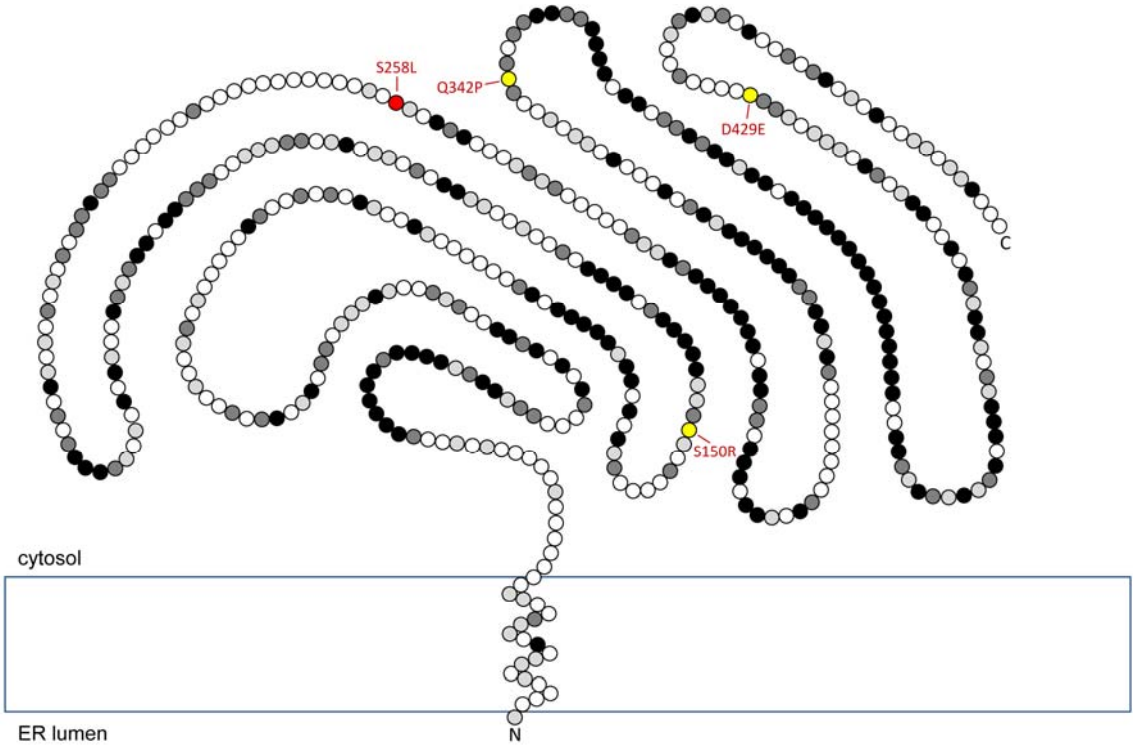

Figure 5. Schematic model of mannosyltransferase 1 (ALG1). Mutations and the conservation of the ALG1 amino acids among the human (NP_061982.3), mouse (NP_663337.2), zebra fish (NP_956161.1), fruit fly (NP_650662.1), nematode (AAC77507.2) and budding yeast (NP_009668.1) proteins were mapped as in Figure 2. Membrane topology prediction of the mannosyltransferase 1 was performed like for the DPAGT1 enzyme (Fig. 4).

Mannosyltransferase 2 (ALG2-CDG, CDG-Ii)

The rarest form of CDG described to date is the ALG2 mannosyltransferase deficiency with a single patient identified (Thiel, et al., 2003). This patient is only mildly affected with developmental delay, seizures, poor vision, coagulopathy and delayed myelinization. Accordingly, it is not possible to draw any conclusion on the severity of ALG2-CDG from the description of this single case.

The α-1,3 mannosyltransferase ALG2 enzyme is 416 amino acid-long and is predicted to form a type-I TM protein (Fig. 6). Like the ALG1 mannosyltransferase, its active site is cytosolically oriented and uses GDP-Man as donor substrate. Two heterozygous mutations were identified in the *ALG2* gene of the index patient (Supp. Table S5). The first induces a frame-shift through a single base deletion (c.1040delG). The effect of the second mutation is unclear, since the point mutation, c.393G>T, results in an amino acid exchange (p.K131N), but also seems to alter the stability of the *ALG2* transcript (Thiel, et al., 2003).

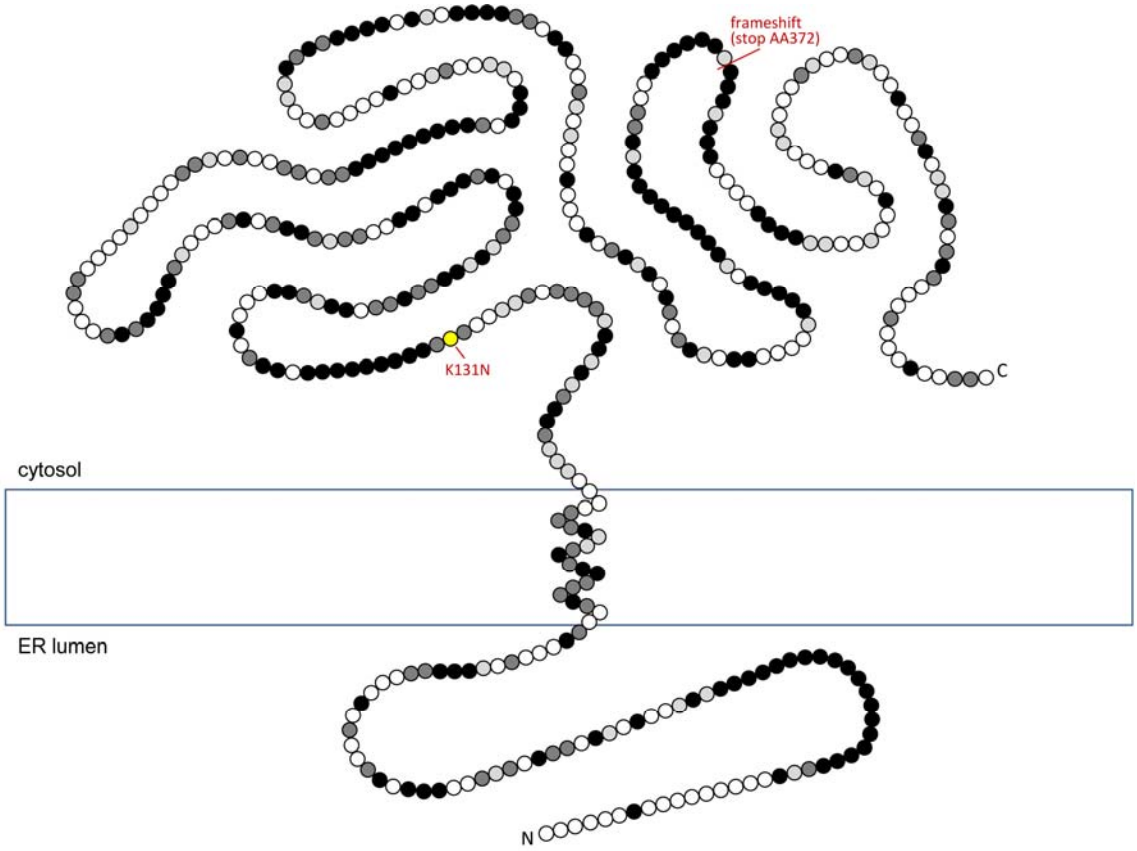

Figure 6. Schematic model of mannosyltransferase 2 (ALG2). Mutations and the conservation of the ALG2 amino acids among the human (NP_149078.1), mouse (NP_064382.3), zebra fish (NP_001098406.1), fruit fly (NP_647772.1), nematode (NP_495010.2) and budding yeast (NP_011450.1) proteins were mapped as in Figure 2. Membrane topology prediction of the mannosyltransferase 2 was performed like for the DPAGT1 enzyme (Fig. 4).

Group 3: Luminally acting mannosyltransferases

In the ER lumen, three mannosyltransferases catalyze the stepwise addition of the last four Man units ending with the formation of dolichol-PP-$GlcNAc_2Man_9$. Those three enzymes are hydrophobic proteins with multiple TM domains and use dolichol-P-Man as donor substrate. All of them have been related to a form of CDG.

Mannosyltransferase 6 (ALG3-CDG, CDG-Id)

The clinical presentation was comparable in most of the eleven ALG3-CDG patients and could be summarized as moderate with mainly neurological symptoms. Accordingly, the patients present with a failure to thrive, psychomotor retardation, epilepsy and microcephaly. Facial dysmorphism, hypotonia and visual impairment are also observed in the majority of the cases

(Stibler, et al., 1995; Denecke, et al., 2005; Schollen, et al., 2005; Sun, et al., 2005a; Kranz, et al., 2007c; Rimella-Le-Huu, et al., 2008). However, a particular feature of this form of CDG constitutes deformations of hands and feet (Denecke, et al., 2005; Schollen, et al., 2005; Sun, et al., 2005a; Kranz, et al., 2007c; Rimella-Le-Huu, et al., 2008), which are not commonly seen in CDG.

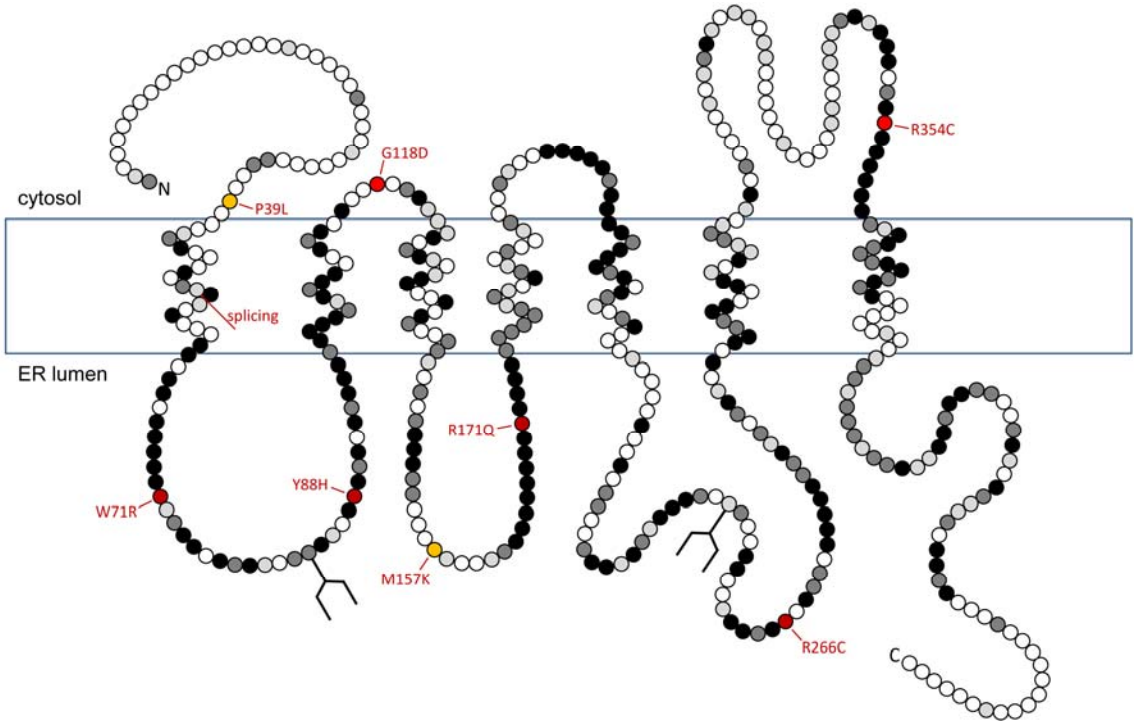

Figure 7. Schematic model of mannosyltransferase 6 (ALG3). Mutations and the conservation of the ALG3 amino acids among the human (NP_005778.1), mouse (NP_666051.2), zebra fish (NP_001018532.1), fruit fly (NP_523829.2), nematode (NP_496950.2) and budding yeast (NP_009471.1) proteins were mapped as in Figure 2. Membrane topology prediction of the mannosyltransferase 6 was performed like for the DPAGT1 enzyme (Fig. 4). Two potential N-glycosylation sites at position p.N83 and p.N253 are shown schematically.

After being flipped into the ER lumen, the dolichol-PP-$GlcNAc_2Man_5$ intermediate is elongated for one Man unit by the *ALG3* encoded α-1,3 mannosyltransferase 6. It is predicted to span the ER membrane seven times within its 438 amino acid sequence (Fig. 7). The asparagines at positions p.N83 and p.N253 represent potential N-glycosylation sites. The TM domains and the loops protruding to the ER lumen display the highest level of conservation among eukaryotes.

The eleven ALG3-CDG cases account for eight point mutations and a single splicing defect (Supp. Table S6) (Stibler, et al., 1995; Körner, et al., 1999; Schollen, et al., 2002; Denecke, et al., 2004; Denecke, et al., 2005; Schollen, et al., 2005; Sun, et al., 2005a; Kranz, et al., 2007c; Rimella-Le-Huu, et al., 2008). Five of these missense mutations lead to substitutions of highly conserved

amino residues within the luminally oriented loops (Fig. 7). Considering their degree of conservation and their orientation with respect to the ER membrane, these mutated amino acids are likely to be involved in the formation of the active center.

Mannosyltransferase 7-9 (ALG9-CDG, CDG-Il)

Only three cases of ALG9-CDG have been characterized to date (Frank, et al., 2004; Weinstein, et al., 2005; Vleugels, et al., 2009b). The corresponding patients presented with typical CDG symptoms such as developmental delay, psychomotor retardation, hypotonia, seizures, hepatomegaly, microcephaly and pericardial effusion. Gastrointestinal problems (Vleugels, et al., 2009b) and bronchial asthma (Frank, et al., 2004) were reported in one case. This form of CDG exhibits no unique feature and can be classified as moderate, although the three cases described to date do not allow drawing a conclusion on the clinical picture.

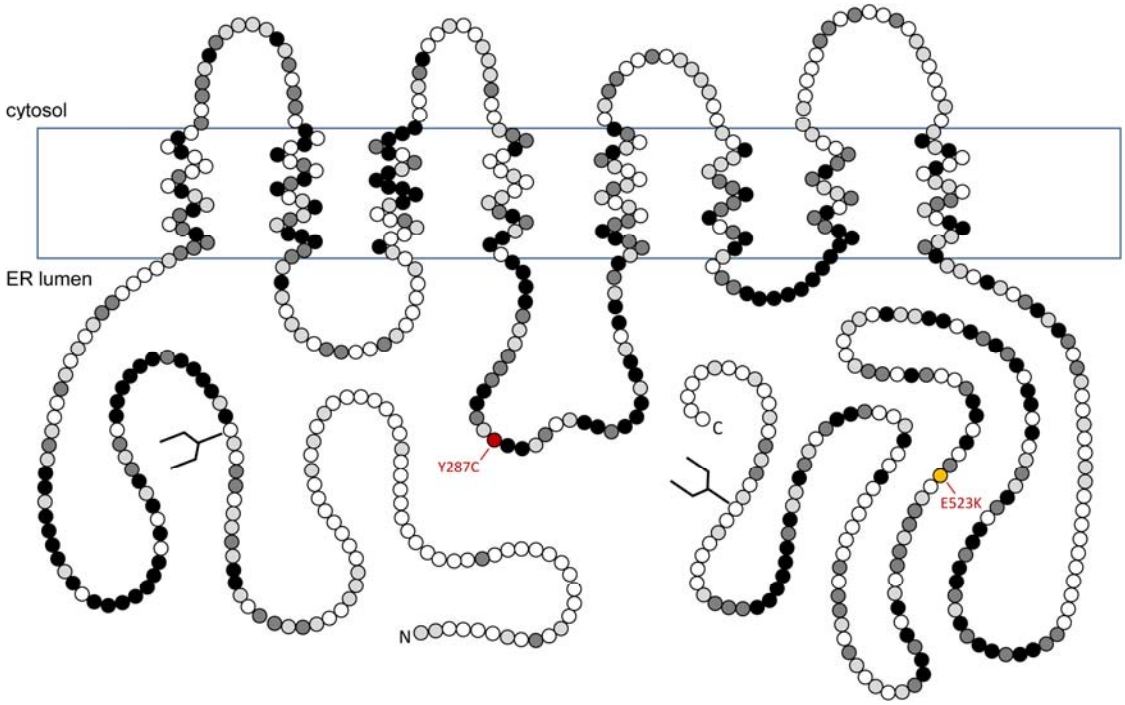

Figure 8. Schematic model of mannosyltransferase 7-9 (ALG9). Mutations and the conservation of the ALG9 amino acids among the human (NP_001071158.1), mouse (NP_598742.1), zebra fish (CAN88585.1), fruit fly (NP_651353.1), nematode (NP_496282.2) and budding yeast (NP_014180.1) proteins were mapped as in Figure 2. Membrane topology prediction of the mannosyltransferase 7-9 was performed like for the DPAGT1 enzyme (Fig. 4). Two potential N-glycosylation sites at position p.N77 and p.N593 are shown schematically.

The two identified mutations (Supp. Table S7) occur homozygously in the *ALG9* gene, which codes for a 611 amino acid α-1,2 mannosyltransferase. The enzyme, which is predicted to include eight TM domains and two N-glycosylation sites, catalyzes the addition of the seventh and the ninth Man of the dolichol-linked oligosaccharide. The mutations p.Y287C and p.E523K lie within conserved loops protruding into the ER lumen (Fig. 8) (Frank, et al., 2004; Weinstein, et al., 2005; Vleugels, et al., 2009b).

Mannosyltransferase 8 (ALG12-CDG, CDG-Ig)

Six of the eight characterized ALG12-CDG patients present a similar set of clinical features, including facial dysmorphism, psychomotor retardation, developmental delay, hypotonia and decreased coagulation factors. Prominent also are respiratory impairment, feeding problems and the absence of seizures, gastrointestinal and hepatic symptoms (Chantret, et al., 2002; Grubenmann, et al., 2002; Thiel, et al., 2002; Di Rocco, et al., 2005; Eklund, et al., 2005b). The low levels of serum immunoglobulin G define a possible indicator for mannosyltransferase 8 deficiency. The overall moderate severity of ALG12-CDG was also supported by the observation that some patients were actually able to walk and showed speech involvement (Eklund, et al., 2005b). However, two ALG12-CDG siblings demonstrate a much more severe disease (Kranz, et al., 2007a). In addition to the symptoms previously mentioned, these two patients presented with skeletal dysplasia, generalized oedema and audiovisual impairment. Both patients died within the first two years of life.

The α-1,6 mannosyltransferase 8, encoded by the human *ALG12* gene, transfers the eighth Man from dolichol-P-Man to the dolichol-linked oligosaccharide. The 488 amino acid protein is predicted to include eleven TM domains (Fig. 9). The enzyme is conserved among eukaryotes, whereas TM domains and the predicted loops oriented to the ER display the highest level of conservation. Two asparagines at positions p.N250 and p.N463 represent consensus sequences for N-glycosylation.

To date, eight ALG12-CDG cases have been described encompassing eleven mutations, whereof nine are missense, one nonsense and a single base deletion causing a frame shift (Supp. Table S8) (Chantret, et al., 2002; Grubenmann, et al., 2002; Thiel, et al., 2002; Di Rocco, et al., 2005; Eklund, et al., 2005b; Kranz, et al., 2007a). Noteworthy, nearly all mutations are mapped to TM domains or to their borders and most of these mutations lead to substitution of highly conserved amino acids (Fig. 9). The siblings, which presented with the more severe clinical features, were compound heterozygous for the p.G101R and the p.R146Q mutations (Kranz, et al., 2007a). While the first mutation is so far unique, the second was already discovered in another case of ALG12-CDG in combination with the p.T67M mutation (Grubenmann, et al., 2002). According to the severe clinical outcome, the p.G101 and the p.R146 residues seem to be essential for ALG12

functionality. This assumption is supported by finding that the glycine at position p.G101 is mainly conserved among eukaryotes, while the arginine at position p.R146 is even strictly conserved in all eukaryotic model organisms examined (Fig. 9).

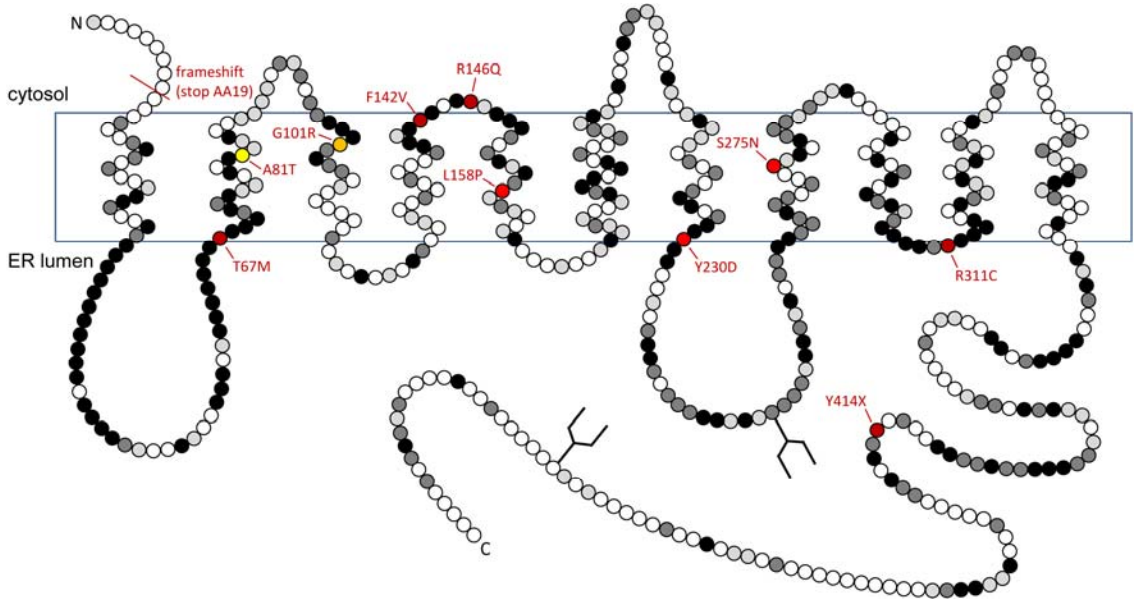

Figure 9. Schematic model of mannosyltransferase 8 (ALG12). Mutations and the conservation of the ALG12 amino acids among the human (NP_077010.1), mouse (EDL04396.1), zebra fish (NP_001092219.1), fruit fly (NP_649939.1), nematode (NP_505071.1) and budding yeast (NP_014427.1) proteins were mapped as in Figure 2. Membrane topology prediction of the mannosyltransferase 8 was performed like for the DPAGT1 enzyme (Fig. 4). Two potential N-glycosylation sites at position p.N250 and p.N463 are shown schematically.

Group 4: Luminally acting glucosyltransferases

The biosynthesis of the dolichol-linked oligosaccharide is completed by the successive addition of three Glc units (Fig. 1). This task is achieved by three ER membrane glucosyltransferases, encoded by the *ALG6*, the *ALG8* and the *ALG10* gene, which utilize dolichol-P-Glc as donor substrate. While defects in the first two enzymes have been associated to CDG, no mutation in the *ALG10* gene has so far been identified.

Glucosyltransferase 1 (ALG6-CDG, CDG-Ic)

The deficiency of ALG6 glucosyltransferase is the second most frequent form of CDG after PMM2-CDG with 36 cases registered so far and representing 20 distinct mutations (Imbach, et al., 1999; Grunewald, et al., 2000; Hanefeld, et al., 2000; Imbach, et al., 2000a; Westphal, et al.,

2000a; Westphal, et al., 2000b; de Lonlay, et al., 2001; Schollen, et al., 2002; Newell, et al., 2003; Westphal, et al., 2003; Sun, et al., 2005b; Vuillaumier-Barrot, 2005; Eklund, et al., 2006). The clinical presentation could be described as mild to moderately severe with psychomotor retardation, developmental delay, seizures, hypotonia, coagulopathy, feeding problems and visual impairment. The occurrence of strong dysmorphic features, gastrointestinal problems or protein-losing enteropathy is rather rare.

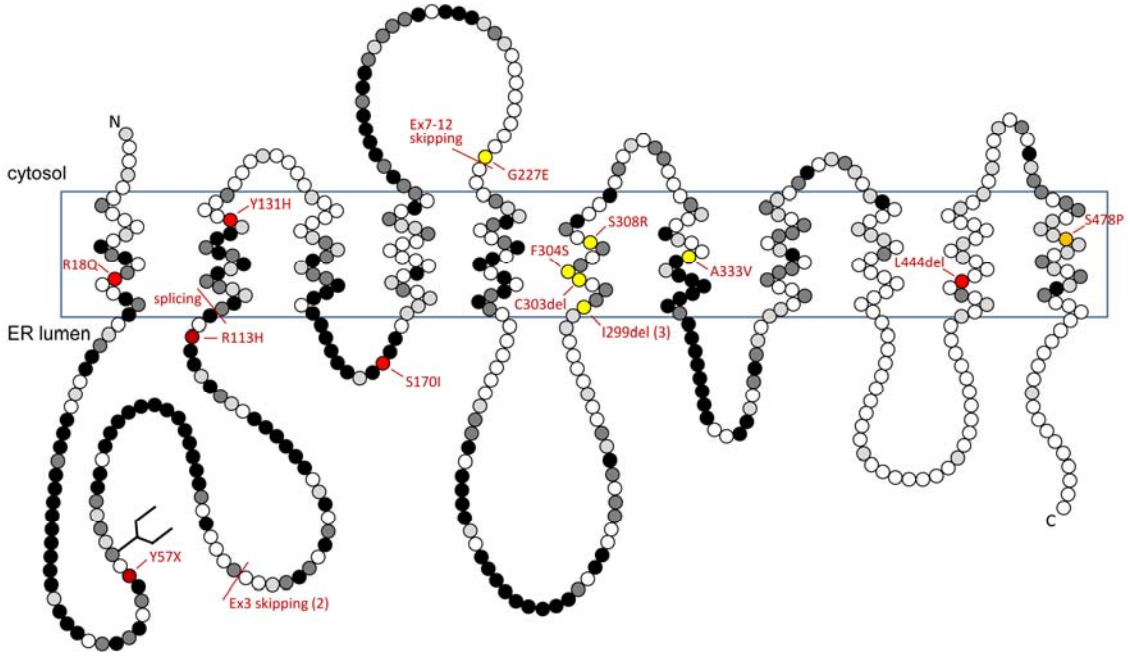

Figure 10. Schematic model of glucosyltransferase 1 (ALG6). Mutations and the conservation of the ALG6 amino acids among the human (NP_037471.2), mouse (NP_001074733.1), fruit fly (NP_609393.1), nematode (NP_495685.1) and budding yeast (NP_014644.1) proteins were mapped as in Figure 2. The protein sequence of *Tetraodon nigroviridis* (CAG11585.1) was used instead of the zebra fish protein, since only a truncated *D. rerio* isoform could be retrieved from the genome database. Membrane topology prediction of the glucosyltransferase 1 was performed like for the DPAGT1 enzyme (Fig. 4). A potential N-glycosylation site at position p.N59 is shown schematically.

The ALG6 α-1,3 glucosyltransferase is 507 amino acid-long and is predicted to span the ER membrane eleven times (Fig. 10). The 20 *ALG6* mutations represent nine missense, one nonsense, four splicing and five deletion mutations (Supp. Table S9). In one case, a portion of the chromosome 1 including the *ALG6* gene is deleted as a *de novo* event (Eklund, et al., 2006). Two of the identified point mutations (p.Y131H and p.F304S) are assumed to be single nucleotide polymorphisms (Vuillaumier-Barrot, et al., 2001; Westphal, et al., 2003). While these single nucleotide polymorphisms do not appear to be pathogenic by themselves, they may lead to

reduced N-glycosylation when combined to other mutations along the pathway of dolichol-linked oligosaccharide assembly (Westphal, et al., 2002). Surprisingly, three of the five deletion mutations lead to an in-frame removal of isoleucine at position p.I299 (Hanefeld, et al., 2000; Westphal, et al., 2000b; Sun, et al., 2005b). The deletion of three consecutive base triplets in three independent patients determines a deletion hotspot. The amino acids surrounding p.I299 are encoded to some extend by the DNA repeat c.382TAATAAT, which might facilitate deletions. The majority of the mutations affect amino acids positioned within the eleven TM domains (Fig. 10). This fact suggests that TM domains might not only play an important role in defining the protein structure, but also in catalysis, presumably through the binding to dolichol-linked substrates. Two missense mutations (p.R113H and p.S170I) change strictly conserved amino residues mapping to ER luminal loops, which might constitute part of the active site (Fig. 10).

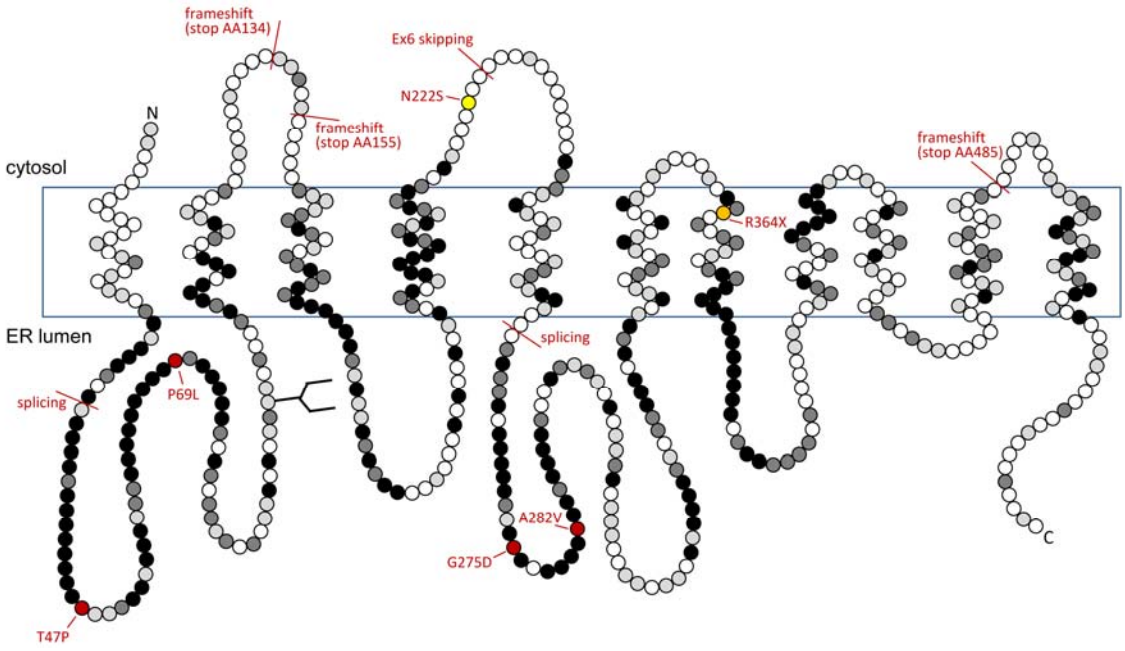

Figure 11. Schematic model of glucosyltransferase 2 (ALG8). Mutations and the conservation of the ALG8 amino acids among the human (NP_076984.2), mouse (NP_950200.2), zebra fish (NP_001017647.1), fruit fly (NP_572355.1), nematode (NP_001021940.1) and budding yeast (NP_014710.1) proteins were mapped as in Figure 2. Membrane topology prediction of the glucosyltransferase 2 was performed like for the DPAGT1 enzyme (Fig. 4). A potential N-glycosylation site at position p.N96 is shown schematically.

Glucosyltransferase 2 (ALG8-CDG, CDG-Ih)

Whereas ALG6 deficiency yields a rather mild form of CDG, mutations in the *ALG8* glucosyltransferase gene lead to a severe form of the disease. Five of nine ALG8 deficient patients died within the first months of life (Schollen, et al., 2004; Eklund, et al., 2005c; Stölting,

et al., 2009; Vesela, et al., 2009). These cases were accompanied by multiple symptoms like strong dysmorphic features, hypotonia, gastrointestinal disorders, hepatomegaly, coagulopathy, oedema, cardio-respiratory problems, protein-losing enteropathy and ascites. Remarkably, neurologic involvement was rather minimal in most of the patients (Chantret, et al., 2003; Schollen, et al., 2004; Eklund, et al., 2005c; Vesela, et al., 2009). However, a pair of siblings displayed a much milder form of ALG8 deficiency. Besides dysmorphic features, they presented with hypotonia, ataxia, mental retardation (Stölting, et al., 2009).

The ALG8 α-1,3 glucosyltransferase is predicted to be an eleven TM domain ER protein with a size of 526 amino acids (Fig. 11). To date, twelve mutations have been described (Supp. Table S10) (Chantret, et al., 2003; Schollen, et al., 2004; Eklund, et al., 2005c; Stölting, et al., 2009; Vesela, et al., 2009). The p.N222S mutation is likely to represent a single nucleotide polymorphism, since the healthy father of a patient is homozygous for this mutation (Schollen, et al., 2004). The other eleven mutations segregate into four missense mutations, one nonsense mutation, three splicing defects and three frame-shift mutations. The missense mutations p.T47P, p.P69L, p.G275D and p.A282V locate to ER luminal domains and lead to substitutions of strictly conserved amino acids (Fig. 11), thereby probably disrupting the enzymatic center. The residual mutations, leading to truncated forms of the ALG8 protein, decrease the catalytic ability of the affected enzyme drastically. This might, at least partly, explain the severe progression of ALG8-CDG.

Group 5: Proteins affecting dolichol-linked carbohydrates

In addition to the cytosolic PMM2 and PMI enzymes and the ER glycosyltransferases, a handful of proteins are also involved in the biosynthesis of the dolichol-linked oligosaccharide required for N-glycosylation. Some of these proteins have an established activity, whereas others represent essential components without clearly assigned functions. By phosphorylating dolichol, the dolichol kinase enzyme enables the transfer of GlcNAc-P to dolichol-P, thereby initiating the biosynthesis of dolichol-linked oligosaccharide (Fig. 1) (Shridas and Waechter, 2006). The products of the *DPM* genes form a trimeric complex that catalyses the synthesis of dolichol-P-Man (Maeda and Kinoshita, 2008). In contrast to the previous enzymes, the function of the MPDU1 and RFT1 proteins can only be predicted. MPDU1 and its hamster ortholog Lec35 have been proposed to be involved in the utilization of the sugar donor substrates dolichol-P-Man and -Glc (Anand, et al., 2001; Schenk, et al., 2001b). The RFT1 protein has been demonstrated to be involved in the translocation of the dolichol-linked $GlcNAc_2Man_5$ intermediate into the ER lumen (Helenius, et al., 2002). Although the proteins included in this group have diverse biological functions, they all yield to a CDG phenotype in case of mutation.

Dolichol kinase (DOLK-CDG, CDG-Im)

Four cases of DOLK-CDG have been reported to date (Kranz, et al., 2007b). The loss of dolichol kinase affects the biosynthesis of dolichol-linked oligosaccharides, therefore it is also classified as a form of CDG. All four reported patients died in early childhood (Kranz, et al., 2007b). This very severe clinical phenotype is marked by hypotonia, skin disorders and the loss of hair. Individual patients also presented with cardiomyopathy, seizures, hypoglycaemia, microcephaly and visual impairment (Kranz, et al., 2007b).

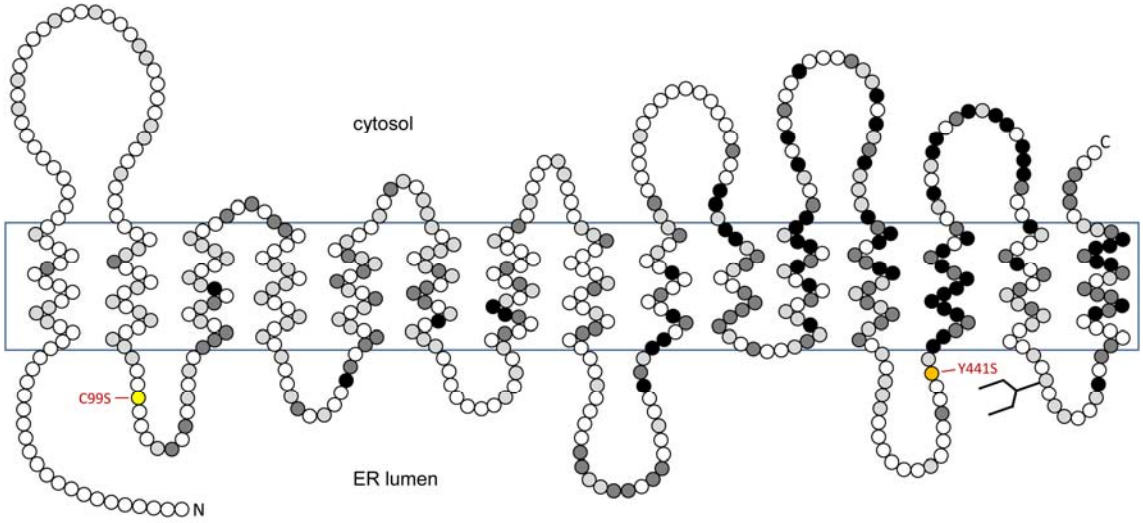

Figure 12. Schematic model of dolichol kinase (DOLK). Mutations and the conservation of the DOLK amino acids among the human (NP_055723.1), mouse (NP_808316.1), zebra fish (NP_001103954.1), fruit fly (NP_611139.1), nematode (NP_001022925.1) and budding yeast (NP_013726.1) proteins were mapped as in Figure 2. The *C. elegans* dolichol kinase is much shorter than the other eukaryotic orthologs, leading to an overall reduced conservation grade within the N-terminal part. Membrane topology prediction of the dolichol kinase was performed like for the DPAGT1 enzyme (Fig. 4). A potential N-glycosylation site at position p.N500 is shown schematically.

The mainly hydrophobic 538 amino acids of the dolichol kinase are predicted to form a 15 TM domain protein, which might be glycosylated at asparagine p.N500 (Fig. 12). This prediction differs slightly with an earlier model, which assigned 13 membrane spanning domains (Shridas and Waechter, 2006). Nevertheless, the N- and the C-termini, as well as the strongly conserved putative CTP-binding pocket (p.S459–p.E474) respect the experimentally proven orientation of the human enzyme regarding the ER membrane (Fig. 12) (Shridas and Waechter, 2006). The level of sequence conservation of the N-terminal region is relatively low, because the *Caenorhabditis elegans* dolichol kinase, with its 281 amino acids, is much shorter than the average dolichol kinases' size in other organisms. This short enzyme overlaps mainly within the

C-terminal part, containing the putative CTP-binding domain (Fig. 12). One of the two homozygously occurring point mutations converts a mainly conserved tyrosine (p.Y441S) in the C-terminal part of the kinase (Supp. Table S11). The other mutation leads to the alteration of a non-conserved cysteine at position p.C99 to a serine, this time in the N-terminal part of the enzyme (Fig. 12). The severe outcome of two trivial point mutations indicates that accurate dolichol kinase function is essential for viability. This statement might not only be due to a defect of N-glycosylation since other functions are assigned to dolichol and dolichol-P (Swiezewska and Danikiewicz, 2005).

Dolichol-P-Man synthase (DPM1-CDG, CDG-Ie and DPM3-CDG, CDG-Io)

Recurrent seizures, hypotonia, developmental delay, dysmorphic features, microcephaly, visual impairment and in some cases ataxia and coagulopathy are the most prominent symptoms found in DPM1-CDG patients (Imbach, et al., 2000b; Kim, et al., 2000; Garcia-Silva, et al., 2004; Dancourt, et al., 2006). The individual cases exhibit certain clinical variations, although all in the range of moderate to severe. The index DPM3-CDG patient described recently (Lefeber, et al., 2009) presented with a very mild phenotype. Except for a mild myopathy, a dilated cardiomyopathy, moderate muscular dystrophy and a single stroke like episode the adult patient is able to lead a virtually normal life.

In humans, dolichol-P-Man synthase is an oligomeric enzyme complex assembled by the *DPM1*, *DPM2* and *DPM3* gene products. So far, the 260 amino acid-long catalytic subunit DPM1 and the tethering polypeptide DPM3 have been described as causes of CDG. DPM2 and DPM3, both ER membrane proteins with each two TM domains and a length of 84 and 92 amino acids, respectively, are required to target the cytosolic DPM1 protein to the ER membrane. While DPM3 interacts directly with DPM1, DPM2 stabilizes the complex by binding to DPM3 (Fig. 13) (Maeda and Kinoshita, 2008).

To date, eight DPM1-CDG patients have been described, which represented six distinct mutations (Supp. Table S12) (Imbach, et al., 2000b; Kim, et al., 2000; Garcia-Silva, et al., 2004; Vuillaumier-Barrot, 2005; Dancourt, et al., 2006). Three of the mutations are deletions or splice defects and lead all to a frame-shift and hence to truncated forms of the enzyme. The three point mutations convert single amino acids of various conservation levels at different sites of the DPM1 protein (Fig. 13). In *DPM3*, the homozygous point mutation p.L85S converts a strictly conserved leucine residue within the terminal coiled-coil domain, which is required for tethering the catalytic DPM1 subunit to the ER membrane (Supp. Table S13) (Lefeber, et al., 2009). Notably, mutations in the DPM complex lead not only to a disorder of N-glycosylation, but also O-mannosylation and glycosylphosphatidylinositol-anchor formation are impaired, since

dolichol-P-Man is utilized as donor substrate for these posttranslational modifications as well (Maeda and Kinoshita, 2008).

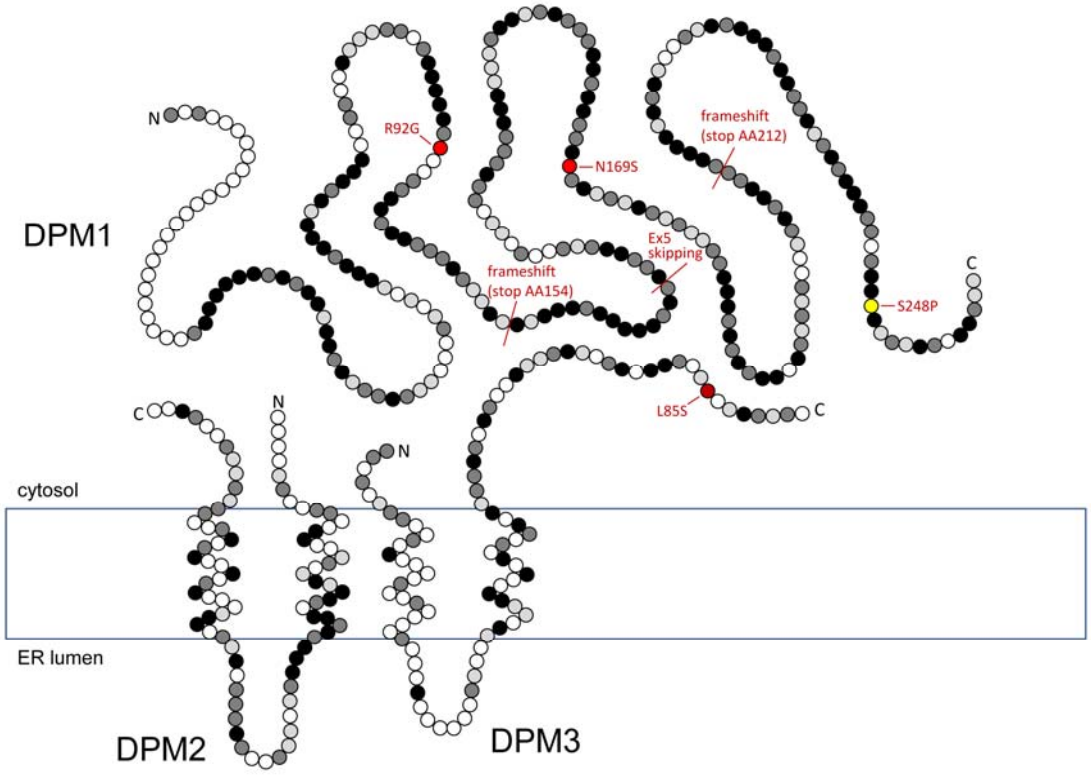

Figure 13. Schematic model of dolichol-P-Man synthase (DPM1/2/3). Mutations and the conservation of the DPM1 amino acids among the human (NP_003850.1), mouse (NP_034202.1), zebra fish (NP_001003596.1), fruit fly (NP_609980.1), nematode (NP_499931.2) and budding yeast (NP_015509.1) proteins were mapped as in Figure 2. Conservation of the DPM2 amino acids among the human (NP_003854.1), mouse (NP_034203.1) and zebra fish (NP_001116318.1) proteins and of the DPM3 amino acids among the human (NP_714963.1), mouse (NP_081043.1), zebra fish (NP_957103.2), fruit fly (NP_001034051.1) and nematode (NP_502366.1) proteins and the DPM3 mutation were also mapped as in Figure 2. Given that the *S. cerevisiae* dolichol-P-Man synthase is a monomeric enzyme (Maeda and Kinoshita, 2008), the DPM2 (NP_595676.1) and DPM3 (NP_596640.1) protein sequences from *Schizosaccharomyces pombe* were used for the particular alignments. Additionally, the aberrant DPM2 sequences of *D. melanogaster* and *C. elegans* were displaced by the DPM2 proteins of *Drosophila ananassae* (EDV40477.1) and *Dictyostelium discoideum* (XP_644349.1), respectively. Membrane topology prediction of the dolichol-P-Man synthase subunits 2 and 3 was performed like for the DPAGT1 enzyme (Fig. 4). The organization of the entire complex was adapted from Maeda *et al.* (Maeda and Kinoshita, 2008).

Man-P-dolichol utilizing defect 1 (MPDU1-CDG, CDG-If)

The clinical outcome of the four described MPDU1-CDG patients is variable. While one patient died in early childhood due to a seizure induced apnea, the others present with mild to moderately severe phenotypes. Shared symptoms are psychomotor retardation, seizures, hypotonia, gastrointestinal problems, visual impairment and, as an atypical hallmark, skin disorders (Kranz, et al., 2001; Schenk, et al., 2001b).

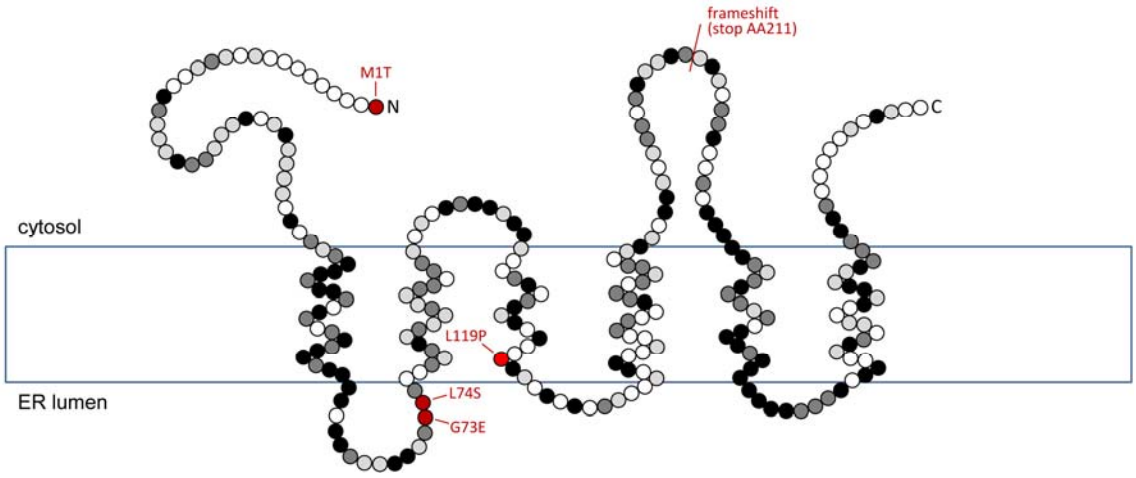

Figure 14. Schematic model of Man-P-dolichol utilizing defect 1 (MPDU1). Mutations and the conservation of the MPDU1 amino acids among the human (NP_004861.2), mouse (NP_036030.2), zebra fish (NP_001002130.1), fruit fly (NP_608889.1) and nematode (NP_505155.1) proteins were mapped as in Figure 2. Since MPDU1 is neither present in *S. cerevisiae* nor in *S. pombe*, the orthologous protein of *Aspergillus niger* (XP_001401680.1) was used for the alignment. Membrane topology prediction of the Man-P-dolichol utilizing defect 1 protein was performed like for the DPAGT1 enzyme (Fig. 4).

The human *MPDU1* gene, whose product is orthologous to the Chinese hamster Lec35 protein, encodes a 247 amino acid ER membrane protein that is predicted to form six TM domains (Fig. 14). Despite its important role in mammalian N-glycosylation, no Lec35 ortholog could be identified in the yeast *S. cerevisiae*. The Man-P-dolichol utilizing defect 1 protein has been proposed to be involved in the lateral distribution of dolichol-P-Man and dolichol-P-Glc within the ER membrane (Schenk, et al., 2001b). It is therefore important for the availability of these sugar substrates to the ER mannosyl- and glucosyltransferases. Accordingly, MPDU1 deficiency leads to the accumulation of dolichol-PP-$GlcNAc_2Man_5$ and dolichol-PP-$GlcNAc_2Man_9$ intermediates (Kranz, et al., 2001; Schenk, et al., 2001b). The four cases of MPDU1-CDG characterized to date revealed five mutations (Supp. Table S14). Four of them are common missense mutations and the fifth is the deletion of a single nucleotide (c.511delC), which leads to a frame-shift (Kranz, et al., 2001; Schenk, et al., 2001b). The mutation of the start methionine in

one of the patient's allele switches the translation start to the next methionine in a different frame and thus leads to the loss of the MPDU1 protein (Schenk, et al., 2001b). The other point mutations affect highly conserved amino acids throughout the protein (Fig. 14).

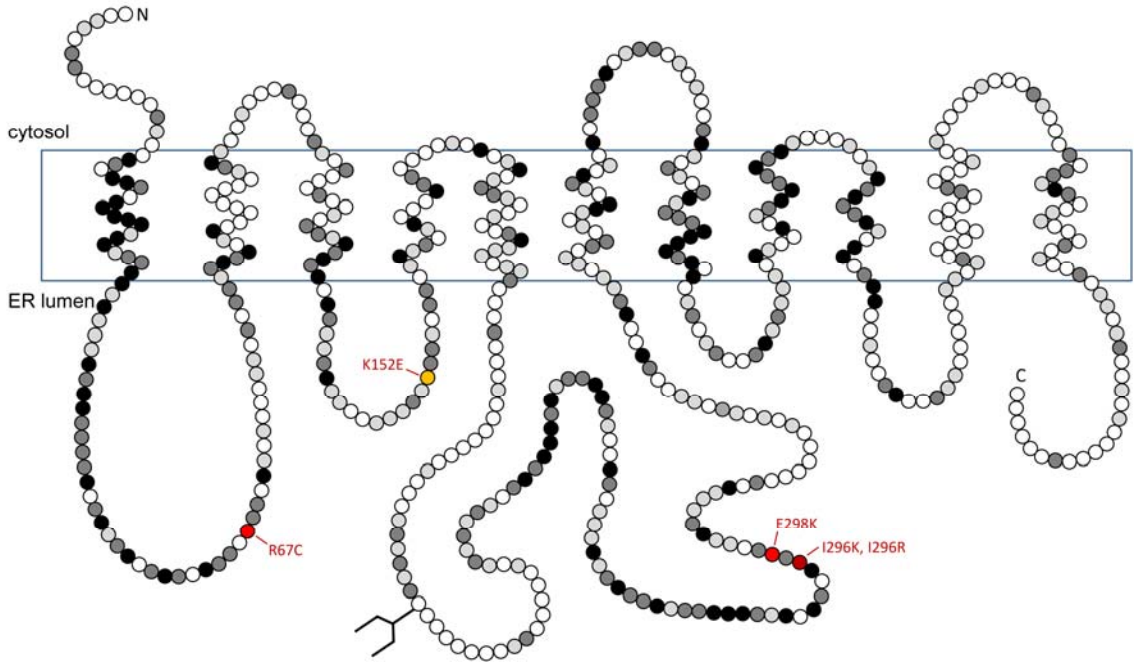

Figure 15. Schematic model of RFT1. Mutations and the conservation of the RFT1 amino acids among the human (NP_443091.1), mouse (NP_808483.2), zebra fish (XP_688354.3), fruit fly (NP_572246.1), nematode (NP_001023610.1) and budding yeast (NP_009533.1) proteins were mapped as in Figure 2. Membrane topology prediction of the RFT1 protein was performed like for the DPAGT1 enzyme (Fig. 4). A potential N-glycosylation site at position p.N227 is shown schematically.

RFT1 (RFT1-CDG, CDG-In)

Mutations in the *RFT1* gene have only been described recently. They lead to the accumulation of dolichol-PP-$GlcNAc_2Man_5$ (Haeuptle, et al., 2008; Vleugels, et al., 2009a). The exact function of the RFT1 protein is currently intensively discussed. It is definitely involved in the translocation of the dolichol-linked oligosaccharide $GlcNAc_2Man_5$ into the ER lumen, but it has still to be demonstrated whether RFT1 is the flippase itself (Helenius, et al., 2002; Frank, et al., 2008). At any rate, mutations in the *RFT1* gene lead to a disorder of N-glycosylation termed RFT1-CDG (Haeuptle, et al., 2008). At least two of the six patients died in childhood, suggesting a severe phenotype. Common clinical features include developmental delay, hypotonia, seizures, feeding problems, dysmorphic features and sensorineural deafness, which could be taken as a characteristic feature. In addition, some patients suffered from coagulopathy, visual impairment and respiratory problems (Haeuptle, et al., 2008; Vleugels, et al., 2009a).

The human RFT1 protein with its 541 amino acids spans the ER membrane eleven times according to prediction algorithms (Fig. 15). The asparagine at position p.N227 might be N-glycosylated. All five identified point mutations convert strongly conserved amino acids (Supp. Table S15) (Haeuptle, et al., 2008; Vleugels, et al., 2009a). Interestingly, one patient is heterozygous for two different point mutations affecting the same nucleotide (c.887T>A/G) and consequently modifying the same amino acid differently (p.I296K/R). All mutated residues are localized to the first three ER luminally-oriented loops of the RFT1 protein. These loops display, together with the TM domains, an overall high conservation among species and might therefore be important for RFT1 functionality (Fig. 15).

Discussion

The comparison of nearly 1000 cases of deficient dolichol-linked oligosaccharide biosynthesis (Table 1) conveys interesting facts regarding the clinical severity of the disease in relation to the position of the genetic defect along the biosynthetic pathway. The biosynthesis of dolichol-linked oligosaccharides is sequential (Kornfeld and Kornfeld, 1985) (Fig. 1), meaning that a block along the pathway will result in the accumulation of an incomplete oligosaccharide. The oligosaccharyltransferase (OST) complex transfers preferentially the complete oligosaccharide to asparagine residues on acceptor proteins, but incomplete oligosaccharides can to some extent be recognized by OST and transferred to proteins, yet to a low efficiency (Körner, et al., 1999; Cipollo, et al., 2001). It is expected that this efficiency decreases with the degree of incompleteness of the dolichol-linked oligosaccharide. Accordingly, deficiency of ALG3 and ALG9 should be more severe than deficiency of ALG6 and ALG8. Furthermore, deficiency of cytosolically active enzymes like ALG1 and ALG2 should be even more severe, because the accumulating dolichol-linked oligosaccharides remain unavailable to the luminally-oriented OST complex. This gradation in the level of N-glycosylation output based on the position of the genetic defect is clearly visible in yeast glycosylation mutants (Huffaker and Robbins, 1982; Kukuruzinska and Robbins, 1987; Jackson, et al., 1993; Stagljar, et al., 1994; Burda and Aebi, 1998). However, such a position effect is not clear-cut when examining human CDG cases. This model would predict that the terminal glucosylation defects seen in ALG6 and ALG8 deficiency would be milder than the mannosylation defects seen in ALG3, ALG9 and ALG12 deficiency, for example. However, the association of ALG8 deficiency with a very severe disease (Schollen, et al., 2004) indicates that even dolichol-linked oligosaccharides lacking the terminal two Glc residues accounts for a profound disorder of N-glycosylation. The severity of ALG8-CDG suggests that protein underglycosylation may not be the only defect underlying the disease. Since the terminal Glc residues of N-glycans are involved in the quality control of glycoprotein folding (Ellgaard and

Helenius, 2003), it is possible that glucosylated dolichol-linked oligosaccharides participate to the regulation of this process, too, as for example through the regulation of glucosidase-II activity (Deprez, et al., 2005). Consequently, alteration of glycoprotein folding might also account for the severity of ALG8-CDG.

The absence of ALG10 defects among CDG cases described to date is surprising. The ALG10 enzyme catalyzes the addition of the third Glc (Burda and Aebi, 1998) of the dolichol-linked oligosaccharide (Fig. 1). Assuming that all defects of dolichol-linked oligosaccharide biosynthesis lead to a disorder of N-glycosylation, ALG10 defects would be expected to present with clinical features typical of CDG. Hence, it is puzzling that no case of ALG10 deficiency has been documented yet. On the other hand, this absence of ALG10 deficiency may be explained if the defect indeed does not significantly impair the process of N-glycosylation, thereby remaining clinically undetectable. At the other side of the scale, it could be that *ALG10* is essential for embryonic development and that even a minor decrease of ALG10 activity may not be compatible with life. To further address this question, it is important to continue screening for new forms of CDG.

The identification of additional mutations and gene defects, especially in the glycosylation genes that have not been associated to CDG yet (Fig. 1), will provide additional evidence on the relationship between the extent of N-glycosylation, the position along the biosynthetic pathway and the severity of the clinical picture. Interestingly, a similar absence of position effect is also observed in the forms of congenital muscular dystrophies that are caused by defects of O-mannosylation. In fact, the clinical severity of the disorders is related to the nature of the mutation rather than by the position of the mutated gene along the O-mannosylation pathway (Godfrey, et al., 2007).

Clinical relevance

The analysis of mutations identified to date shows that 150 of the total 203 genetic alterations (Table 1) are missense mutations, whereas 112, i.e. 75%, of these mutations affect highly conserved amino acid residues among eukaryotic proteins. Since conserved amino acids are usually parts of functional motifs (Kinch and Grishin, 2002), it can be assumed that most CDG mutations alter the functions of the affected proteins. Accordingly, mutations of little conserved amino acids would yield minor enzymatic deficiencies that may be accompanied by mild symptoms or even remain asymptomatic. Along this line, it can be predicted that proteins showing the highest degree of sequence conservation among eukaryotes are likely to be more often associated with a disease phenotype. In case of dolichol-linked oligosaccharide biosynthesis, the PMM2 protein is by far the most conserved protein of the pathway (Fig. 2). Supporting this fact, half of the known mutations (103 out of 203) affect the *PMM2* gene (Table

1). However, other proteins of the pathway also show a high degree of sequence conservation among eukaryotes, as for example the MPI and DPAGT1 proteins (Fig. 3 and 4). Yet, only three mutations have been found in the *DPAGT1* gene so far, indicating that sequence conservation alone does not account for the incidence of a genetic disease.

By examining the occurrence of *PMM2* mutations, Schollen *et al.* (Schollen, et al., 2000b) noticed that the p.R141H mutation is very prevalent in the European population with a carrier frequency of about 1/70. Since this mutation is never found at the homozygous state in CDG patients (Matthijs, et al., 1998) due to its inactivating properties on PMM2 activity, it can only be assumed that a selection pressure accounts for the maintenance of this mutation in human populations. However, the nature of this selection pressure is unknown and one can only speculate as to whether a reduction of N-glycosylation is related to reducing the susceptibility to pathogens binding N-glycans as receptors. Such arguments are beyond the scope of this review, but they certainly provide exciting points of reflection when discussing the biological and evolutionary relevance of glycosylation disorders.

Future Prospects

The analysis of mutations in genes involved in dolichol-linked oligosaccharide biosynthesis shows that they mainly affect conserved amino acid residues, thereby impairing protein function. Since a complete loss of protein function is usually not compatible with life, as seen in mice lacking PMM2, MPI and DPAGT1 activity (Marek, et al., 1999; DeRossi, et al., 2006; Thiel, et al., 2006), the mutations encountered in CDG certainly enable a significant level of N-glycosylation output. At the threshold of normal N-glycosylation, the transferrin IEF test may be not sensitive enough to detect N-glycosylation defects caused by mild mutations. The study of these mutations may represent the next challenge in CDG research, since mild disorders of glycosylation are often associated to mild neurological presentations such as slight mental retardation (Giurgea, et al., 2005). The development of new sensitive tests will certainly contribute to determine the true incidence of CDG and to better understand the physiological impact of N-glycosylation.

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Results

1 Human RFT1 Deficiency Leads to a Disorder of N-linked Glycosylation

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Contribution:

LLO analysis
NLO analysis
Mutation analysis
Cloning of human cDNA
Lentiviral complementation

Human RFT1 deficiency leads to a disorder of N-linked glycosylation

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Running title: RFT1 deficiency in CDG

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N-linked glycosylation is an essential post-translational modification of proteins in eukaryotes. The substrate of N-linked glycosylation, dolichol pyrophosphate (DolPP)-$GlcNAc_2Man_9Glc_3$ is assembled through a complex series of ordered reactions requiring the translocation of the intermediate DolPP-$GlcNAc_2Man_5$ structure across the endoplasmic reticulum membrane. A young patient diagnosed with a congenital disorder of glycosylation, characterized by an intracellular accumulation of DolPP-$GlcNAc_2Man_5$, was found to carry a homozygous point mutation in the *RFT1* gene. The c.199C>T mutation introduced the amino acid substitution p.R67C. The human RFT1 protein shares 22% identity with its yeast homolog, which is involved in the translocation of DolPP-$GlcNAc_2Man_5$ from the cytosolic into the lumenal side of the endoplasmic reticulum. Despite the low sequence similarity between the yeast and human RFT1 proteins, we demonstrated both their functional orthology, and the pathologic effect of the human p.R67C mutation by complementation assay in *Δrft1* yeast cells. The causality of the RFT1 p.R67C mutation was further established by restoring normal glycosylation profiles in patient derived fibroblasts after lentiviral expression of a normal *RFT1* cDNA. The definition of the RFT1 defect establishes the functional conservation of the DolPP-$GlcNAc_2Man_5$ translocation process in eukaryotes. RFT1 deficiency in both yeast and human cells leads to the accumulation of incomplete DolPP-$GlcNAc_2Man_5$ and to a profound glycosylation disorder in humans.

N-linked glycosylation is an ubiquitous posttranslational modification of proteins in eukaryotes. N-glycans convey essential signals for the folding and intracellular trafficking of glycoproteins (Helenius and Aebi, 2004). N-glycans also influence the clearance of circulating glycoproteins (Mi, et al., 2002) and the stability of signaling proteins at the cell surface (Chantret, et al., 2003). A particular feature of N-glycans is that they are first assembled in the endoplasmic reticulum (ER) as lipid-linked oligosaccharides (LLO). This assembly proceeds through the sequential addition of monosaccharides to the growing LLO. The process begins by the addition of GlcNAc monophosphate to the lipid carrier dolichol phosphate (DolP) and ends with the formation of DolPP-$GlcNAc_2Man_9Glc_3$. The oligosaccharide is transferred to selected asparagine residues of nascent glycoproteins (Burda and Aebi, 1999). The assembly of LLO requires glycosyltransferases and their respective nucleotide- and dolichol-activated mono-saccharide substrates, but also several proteins that regulate the complex topology of the process. For example, the MPDU1 protein makes the donor substrates DolP-Man and DolP-Glc available for the completion of the LLO beyond DolPP-$GlcNAc_2Man_5$ (Anand, et al., 2001; Kranz, et al., 2001; Schenk, et al., 2001b). Similarly, it was previously shown that in yeast the Rft1 protein is essential for the translocation of the cytosolically oriented intermediate DolPP-$GlcNAc_2Man_5$ into the ER lumen, where LLO assembly is completed (Helenius, et al., 2002).

The pathway of LLO assembly is strongly conserved among eukaryotes. Homologous genes can be found from yeasts to humans for all glycosyltransferases involved. However, it is unclear whether the same degree of conservation applies to the accessory proteins. In fact, whereas *MPDU1* homologs can be found in metazoan and plant genomes, no orthologous gene can be identified in the yeast genome. In the case of *RFT1*, only genes with limited sequence similarity can be retrieved from the genome of higher organisms, thus casting doubt upon the functional significance of RFT1 in the assembly of LLO in general.

The identification of N-linked glycosylation disorders in humans, often referred to as congenital disorders of glycosylation (CDG), has demonstrated the conservation of the LLO assembly pathway between yeasts and humans (Aebi and Hennet, 2001). The expression of human glycosyltransferase genes in yeasts exhibiting the same glycosylation defects demonstrated both the functional orthology of the glycosyltransferases studied and the pathological effect of the mutations identified in CDG cases (Imbach, et al., 1999; Grubenmann, et al., 2002; Frank, et al., 2004; Grubenmann, et al., 2004).

In spite of the extensive use of the yeast as a road map, many cases of CDG have remained untyped. Clinically, these cases present the symptoms typically seen in CDG patients, *i.e.* neurological abnormalities, failure to thrive and varying degrees of dysmorphism (Schenk, et al., 2001b; Wu, et al., 2003). In the present study, we have identified a novel glycosylation defect in such an untyped CDG case, which establishes the importance of the RFT1 protein in human N-linked glycosylation.

Materials and methods

LLO and NLO analysis - The investigation of the patient material was approved by the Ethical Commission of the Kanton Zürich. Fibroblasts were grown in DMEM (Gibco) containing 25 mM Glc and 10% FCS until 90% of confluence. At this point, the fibroblasts were rinsed in PBS and incubated in Glc-/FCS-free DMEM for 45 min, then labeled by adding 150 µCi of [^{3}H]-Man (54.0 Ci/mmol, Amersham Bioscience) for 60 min. LLO and N-linked oligosaccharides (NLO) were isolated from labeled fibroblasts by chloroform/ methanol/water extraction as described previously (Grubenmann, et al., 2004). Oligo-saccharides were released from LLO by mild acid hydrolysis and from NLO by N-glycosidase F (New England BioLabs) digestion (Grubenmann, et al., 2004) and subjected to HPLC (Zufferey, et al., 1995).

Mutation analysis – Total RNA and genomic DNA were isolated from 2 x 10^7 fibroblasts and 5 ml blood samples, respectively, using the TRIzol LS reagent (Invitrogen) according to the manufacturer's instructions. The human *RFT1* cDNA was prepared from 2 µg of total RNA using the primer 5'-GGGCTTTTGGTCTTCACT-3' and 2 units of Omniscript reverse transcriptase (Qiagen). The 20 µl reaction mixtures were incubated at 37 °C for 1 h. The protein-coding region of the human *RFT1* cDNA was amplified by PCR from 2 µl of RT product with the primers 5'-GGCGGCATTTCCTGGTGTCT-3' and 5'-TGGCACTC-TCTGGTGCCTCATC-3'. The exon 3 of the human *RFT1* gene was amplified by PCR from 50 ng of genomic DNA with the primers 5'-GGGCAATTCAGCTTTAGG-3' and 5'-CACCACCAG-TGGTTTATG-3'. The PCR products were sequenced (Synergene Biotech, Switzerland) after removal of the unincorporated nucleotides with QIAquick columns (Qiagen).

Plasmid construction - For construction of the lentiviral expression vector, the human *RFT1* cDNA was subcloned as a PCR fragment flanked by *SpeI* and *XhoI* restriction sites into the *NheI* and *SalI* sites of the pLenti6-*EGFP* plasmid (Invitrogen), thus yielding the pLenti6-*hRFT1* vector. The yeast *rft1* gene with promoter and terminator sequences was amplified from *S.cerevisiae* genomic DNA by PCR and ligated into YCp*lac*33 (Gietz and Sugino, 1988; Kastaniotis, et al., 2004) using the inserted 5'-*PstI* and 3'-*BamHI* restriction sites to generate YCp*lac*33-*ScRFT1*. The pTSV30A-*ScRFT1* (Gietz and Sugino, 1988; Kastaniotis, et al., 2004) plasmid was obtained by subcloning *ScRFT1* from YCp*lac*33-*ScRFT1* using *SacI* and *BamHI* restriction sites. Plasmid YCp*lac*33-pGAL1 was constructed by amplification of the *GAL1* promoter from pYES2 (Invitrogen) and ligation of the PCR product into YCp*lac*33 using the inserted restriction sites 5'-*HindIII* and

3'-*XbaI*. The human *RFT1* cDNA was obtained via the EST clone IMAGE: 6422683 (Geneservice, UK). For construction of the plasmid YCp*lac*33 pGAL1-*hRFT1*, the human *RFT1* ORF was amplified from the IMAGE clone by PCR and cloned into plasmid YCp*lac*33 pGAL1 using the inserted restriction sites 5'-*XbaI* and 3'-*SacI*. The mutant *RFT1[R67C]* cDNA from the CDG patient was subcloned into YCp*lac*33 pGAL1 *hRFT1,* resulting in YCp*lac*33-pGAL1-*hRFT1[R67C].*

Complementation of *rft1Δ* yeast mutants - Yeast strains W1536 5B (*MAT*a, *ade2*Δ, *ade3*Δ, *can1-100*, *his3-11,15*, *leu2-3,112*, *trp1-1*, *ura3-1*) and W1536 8B (isogenic *MAT*α strain) have been described earlier (Kastaniotis, et al., 2004). The diploid W1536a/α was obtained by mating W1536 5B and W1536 8B. W1536 5B-*rft1*Δ was generated by introducing the *rft1::KanMX4* gene replacement cassette into W1536a/α and selecting for geneticin resistance, followed by tetrad dissection on a Singer MSM manual dissection microscope (Singer Instrument). Haploid W1536 5B-*rft1*Δ mutant cells carry plasmid-borne *rft1* to be viable. The yeast cells were grown on either rich YPD (1% yeast extract, 2% peptone and 2% D-glucose), YPGalD (1% yeast extract, 2% peptone, 2% D-galactose and 0.05% D-glucose), YPR (1% yeast extract, 2% peptone and 2% D-raffinose) or synthetic complete media (Sigma or QBiogene) lacking one or more nutrients. The colony-sectoring method has been described by Bender *et al.* (Bender and Pringle, 1991). CPY western blotting was performed as described elsewhere (Burda, et al., 1996).

Lentiviral mediated *RFT1* expression - HEK293T cells (3 x 10^6) were transfected with 20 µg of pLenti6-*hRFT1* and 36 µg of the packing plasmid mix (Invitrogen) using calcium-phosphate precipitation. Eight hour post transfection, the medium was replaced with fresh DMEM containing 10% FCS. The cell supernatant was collected after 48 h and lentiviruses were harvested by centrifugation at 3000 x g for 5 min and filtration through 0.45 µm membranes (Schleicher & Schuell, Germany). CDG and healthy control fibroblasts were infected with recombinant lentiviral particles including the human *RFT1* cDNA or the *EGFP* gene as control. Infected cells were selected with 5 µg/ml blasticidin (Invitrogen) for 10 days.

Results

A young girl was diagnosed with a disorder of N-linked glycosylation based on the detection of abnormal isoelectric focusing of serum transferrin (Stibler, et al., 1998). The patient, designated by the abbreviation KS (Imtiaz, et al., 2000), showed symptoms often encountered in CDG, namely a marked developmental delay, hypotonia, seizures, hepatomegaly and coagulopathy (Imtiaz, et al., 2000). Phosphomannomutase and phospho-mannose isomerase deficiencies were ruled out by enzymatic testing (data not shown). To determine whether the glycosylation disorder was related to a defect of LLO assembly, we analyzed the LLO composition in healthy control and the patient fibroblasts. The LLO profile of control fibroblasts was dominated by the full-length DolPP-$GlcNAc_2Man_9Glc_3$ (Fig. 1A). By contrast, the profile of the CDG patient was marked by an accumulation of the intermediate LLO DolPP-$GlcNAc_2Man_5$, whereas the complete LLO DolPP-$GlcNAc_2Man_9Glc_3$ was strongly reduced (Fig. 1B). The analyses of NLO produced after 1 h labeling of healthy control and CDG fibroblasts with [^{3}H]-Man were indistinguishable. Both profiles showed peaks corresponding to $GlcNAc_2Man_8$ and $GlcNAc_2Man_9$ oligosaccharides (Fig. 1C, D), which are normally found on glycoproteins after trimming of the N-linked glycans by the ER glucosidases-I, –II and ER mannosidase. The absence of $GlcNAc_2Man_5$ in the NLO profile of the CDG patient suggested that only the full-length LLO DolPP-$GlcNAc_2Man_9Glc_3$ was transferred to glycoproteins. This phenotype was reminiscent of the LLO/NLO profiles described in yeast depleted

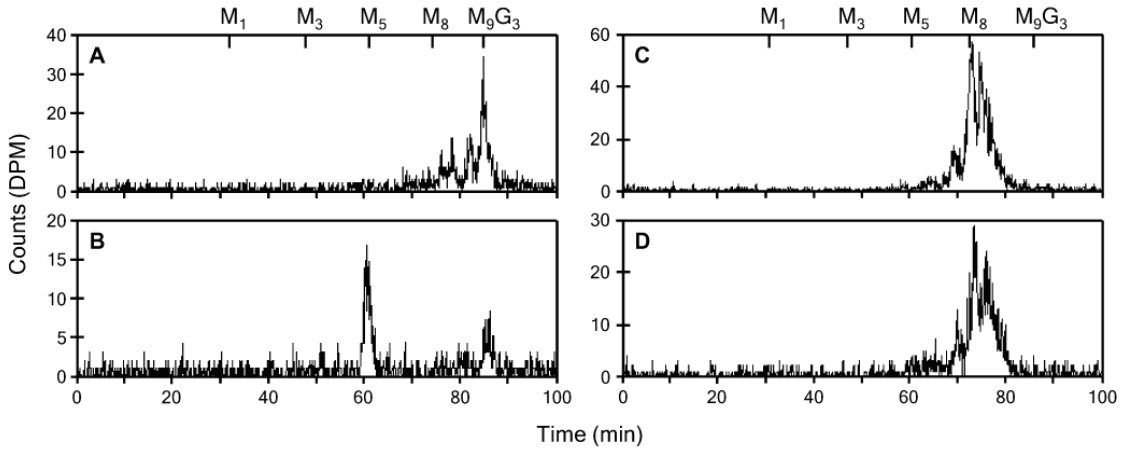

Figure 1. Lipid-linked and N-linked oligosaccharide profiles. The LLO isolated from healthy **(A)** and CDG **(B)** fibroblasts were separated by HPLC, demonstrating the abnormal accumulation of the LLO DolPP-$GlcNAc_2Man_5$ in the CDG sample. The NLO isolated from healthy **(C)** and CDG **(D)** fibroblasts were identical, showing that complete oligosaccharides were transferred to nascent proteins in the CDG cells. The retention times of DolPP-$GlcNAc_2Man_1$ (M_1) to DolPP-$GlcNAc_2Man_9Glc_3$ (M_9G_3) derived from a yeast standard LLO are marked at the top of the profiles.

for the Rft1 protein (Helenius, et al., 2002). In conditions with limiting RFT1 activity, DolPP-$GlcNAc_2Man_5$ accumulates at the cytosolic side of the ER membrane, whereas the small amounts of flipped oligosaccharide are extended to DolPP-$GlcNAc_2Man_9Glc_3$ and transferred to proteins. This results in the underglycosylation of N-glycoproteins.

The search for a human homolog to the yeast Rft1 protein pointed to a single gene, GenBank# BC043595, which encoded a protein of 541 amino acids sharing 22% identity with yeast Rft1. Sequencing of the BC043595 cDNA in the CDG fibroblasts revealed a C to T transition at nucleotide position 199 (Fig. 2A). This point mutation led to the amino acid substitution p.R67C in the human RFT1 protein homolog. The analysis of the BC043595 gene in the DNA of the CDG patient's parents confirmed the heterozygosity (Fig. 2B). The p.R67C mutation was localized to a 50 amino acid-long hydrophilic stretch in the overall hydrophobic Rft1 protein homolog (Fig. 2C).

Genes encoding proteins with varying degrees of similarity to the human RFT1 protein can be retrieved from all eukaryote genomes analyzed, for which the sequence identity ranges from 87% for the mouse homolog down to 17% for fungal homologs (Fig. 3). Although the overall identity is limited, some regions of RFT1 are strongly conserved across species. It is noteworthy that the region comprising the p.R67C mutation was strongly conserved and several arginine residues, including R67 in the human protein, were found in all sequences analyzed (Fig. 3), suggesting the importance of these amino acids for proper functionality.

Growth of the yeast strain W1536-5B-*rft1Δ* relies on the presence of the yeast *RFT1* gene on the plasmid pTSV30A-*ScRFT1*. Cells of this strain background carrying this plasmid develop a red pigment, due to the presence of the *ADE3* gene on pTSV30A, whereas loss of the plasmid results in white cells (Bender and Pringle, 1991). Accordingly, W1536-5B-*rft1Δ*/pTSV30A-*ScRFT1* forms colonies that are uniformly red. This strain was transformed with the additional plasmid YCp*lac*33pGAL1-*hRFT1* that leads to the expression of the human *RFT1* cDNA controlled by the galactose-inducible GAL1 promoter.

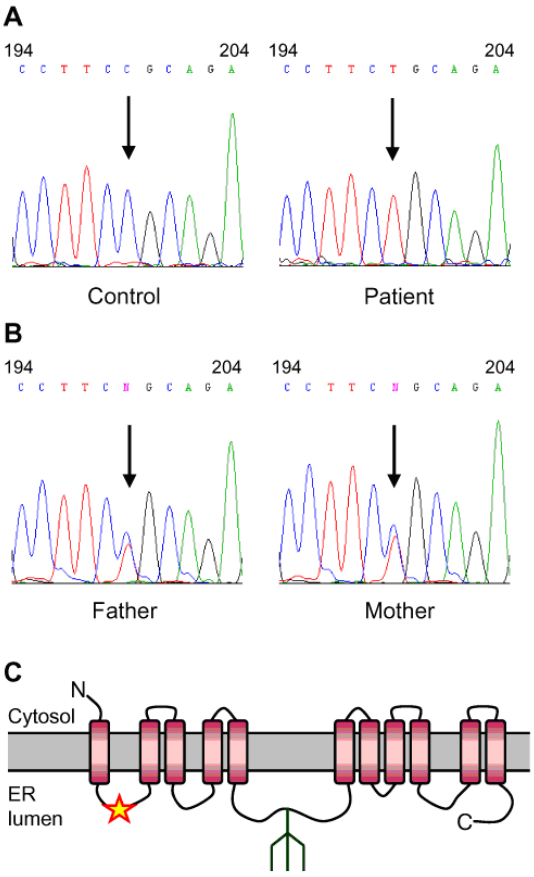

Figure 2. Mutation analysis of human RFT1 in CDG. Electropherograms of *RFT1* cDNA surrounding nucleotide position 199, marked by an arrow, sequenced from normal control and patient cDNA **(A).** The same region was sequenced from the genomic DNA of the CDG patient's parents **(B)**, showing the heterozygozity for the c.199C>T mutation. **(C)** The resulting p.R67C substitution, marked with a star, was predicted to be localized in a hydrophilic loop within the highly hydrophobic RFT1 protein. A potential N-glycosylation site detected at position N227 is shown schematically.

Such cells form colonies that contain white sectors due to the fact that the pTSV30A-*ScRFT1* plasmid was no longer essential for growth and the plasmid could be lost, as visualized by the white sectors. This indicated functional complementation of the *rft1* defect by expression of the human *RFT1* cDNA (Fig. 4A). This experiment demonstrated that the human cDNA investigated does indeed encode the orthologous protein to yeast Rft1. The same experiment performed with a plasmid expressing the CDG *RFT1[R67C]* allele did not yield any sectoring colonies (Fig. 4A), thus demonstrating that the p.R67C mutation led to reduced function even in the yeast system. As shown previously (Helenius, et al., 2002), Rft1 depletion in yeast leads to the underglycosylation of the vacuolar N-linked glycoprotein carboxypeptidase Y. When W1536 5B-*rft1Δ* yeasts were complemented with YCplac33 pGAL1-*hRFT1*, a normal carboxy-peptidase Y glycosylation profile was restored (Fig. 4B), thus showing that the effect of the human RFT1 on *rft1Δ* yeast was indeed related to N-glycosylation.

Finally, we introduced a normal human *RFT1* cDNA in the fibroblasts of the CDG patient to demonstrate that the glycosylation disorder was the consequence solely of the *RFT1* mutation identified. Healthy control and CDG fibroblasts were infected with recombinant lentiviruses expressing either the normal *RFT1* cDNA or *EGFP* as a negative control. The analysis of LLO profiles in the infected fibroblasts showed an increased formation of the full-length DolPP-$GlcNAc_2$-Man_9Glc_3 and a reduced presence of DolPP-$GlcNAc_2Man_5$ (Fig. 5A), whereas the *EGFP* expression control had no influence on the LLO profile (Fig. 5B). The expression of either normal human *RFT1* or *EGFP* in control fibroblasts had no effect on the LLO profiles (data not shown).

Discussion

The identification of a human glycosylation disorder associated with an RFT1 defect underlines the functional conservation of the RFT1 protein in eukaryotes. In spite of a limited sequence similarity between the yeast and the human RFT1 proteins, RFT1 deficiency led to identical biochemical phenotypes in both species, *i.e.* to an accumulation of the LLO DolPP-$GlcNAc_2Man_5$, underglycosylation and thus to a

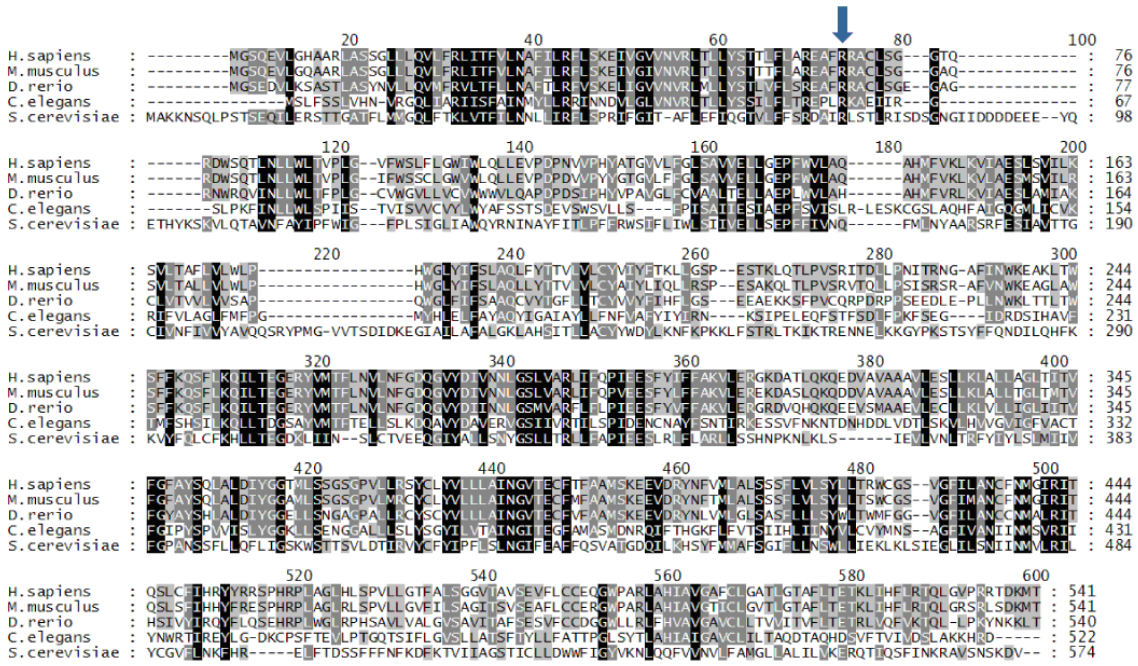

Figure 3. RFT1 protein sequence comparison. Amino acid sequences of proteins derived from *Homo sapiens*, *Mus musculus*, *Danio rerio*, and *Caenorhabditis elegans* showing similarity to the *Saccharomyces cerevisiae* RFT1 protein as performed by ClustalW analysis (Thompson, et al., 1994). Residues conserved in all five species are shown in black. The R residue at position 67 in the human RFT1 protein (see arrow) is conserved in the five species. Total amino acid identity with the human sequence ranged from 87% for the mouse protein down to 22% for the *S. cerevisiae* Rft1 protein.

severe disease in humans. Clinically, the RFT1-deficient CDG patient presented with symptoms frequently encountered in CDG patients, namely failure to thrive, psychomotor retardation, seizures, hypotonia and coagulopathy. Among the various types of CDG, the clinical severity of the RFT1 deficiency resembled the diseases caused by ALG3 and DPM1 deficiencies, also known as CDG-Id (Körner, et al., 1999) and CDG-Ie (Imbach, et al., 2000b; Kim, et al., 2000), respectively. The *ALG3* gene encodes the DolP-Man dependent mannosyltransferase that catalyzes the elongation of LLO from DolPP-$GlcNAc_2Man_5$ to DolPP-$GlcNAc_2Man_6$ (Aebi, et al., 1996). The DPM1 protein is the catalytic subunit of the DolP-Man synthase complex (Maeda, et al., 2000). In CDG-Ie, the shortage in DolP-Man impairs the elongation of the LLO DolPP-$GlcNAc_2Man_5$ in the ER lumen. Although DPM1, ALG3 and RFT1 defects all lead to the accumulation of the LLO DolPP-$GlcNAc_2Man_5$, their impact on protein N-glycosylation is expected to be different. In fact, RFT1 defect yields complete LLO structures for the transfer to proteins, yet in limited amount, whereas ALG3 and DPM1 defects yield low amounts of complete LLO combined with the lumenal accumulation of the DolPP-$GlcNAc_2Man_5$. The clinical similarity between these three glycosylation defects suggests that the limited availability of complete LLO alone dictates the extent of the clinical manifestations. In the case of DPM1 deficiency, the decreased DolP-Man availability is expected to also affect O-mannosylation and GPI-anchor formation. Yet, the comparison with the clinical features of ALG3 and RFT1 deficiency suggests that the majority of the symptoms are rather due to abnormal N-glycosylation.

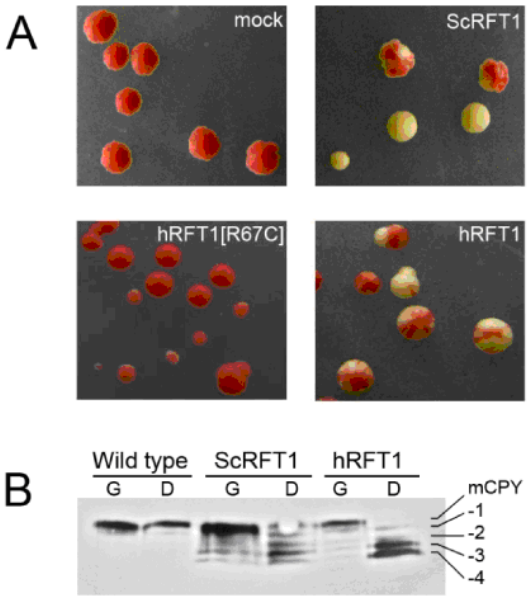

Figure 4. Complementation of *rft1*Δ yeasts. W1536 5B *rft1*Δ/ pTSV30A-*ScRFT1* cells trans-formed with a plasmid carrying a complementing *RFT1* variant will form sectored colonies as they are allowed to lose the red pigment –inducing pTSV30A -*ScRFT1* plasmid. Cells were trans-formed with: YCp*lac*33 (mock), YCp33 *GAL ScRFT1*, YCp33 *GAL hRFT1[R67C]* and YCp33 *GAL hRFT1* **(A)**. Western blotting analysis of the yeast strain W1536 *rft*Δ transformed with yeast and human *RFT1* expressed from the yeast *GAL1* promoter showed a near wild type levels of carboxypeptidase Y glycosylation when grown on 2 % Gal [G], and an accumulation of under-glycosylated isoforms when *RFT1* expression was repressed by 4% Glc [D] **(B).** Carbon source did not affect carboxypeptidase Y glycosylation in wild type yeast (left panel).

The functional conservation of the yeast and human RFT1 proteins emphasizes the essential role of RFT1 in lower and higher eukaryotes. However, the function of the RFT1 protein still remains unclear. The contribution of RFT1 to the specific translocation of DolPP-$GlcNAc_2Man_5$ suggests that it may function as a flippase (Helenius, et al., 2002), although such an activity could not be confirmed *in vitro*. A similar uncertainty relates to the function of the MPDU1 protein (Anand, et al., 2001), which is involved in making DolP-Man and DolP-Glc substrates available to ER lumenal mannosyl- and glucosyltransferases. It is presently unclear whether MPDU1 affects the flipping of these substrates across the ER membrane or whether MPDU1 affects their local concentration by a different mechanism.

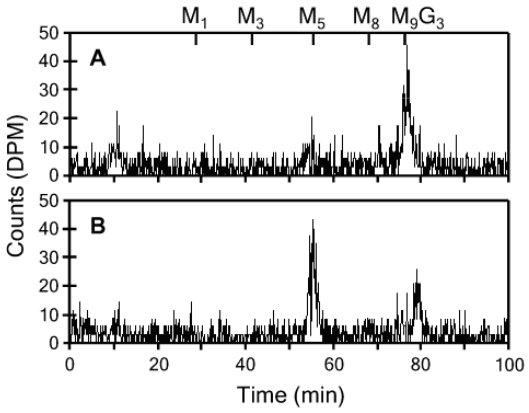

Figure 5. Lentiviral mediated complementation in CDG fibroblasts. The LLO profiles of fibroblasts infected with recombinant lentiviruses expressing either human *RFT1* **(A)** or *EGFP* as negative control **(B)** were analyzed. The profiles show that the expression of the normal *RFT1* cDNA in CDG fibroblasts restored the synthesis of the complete LLO DolPP-$GlcNAc_2Man_9Glc_3$. The expression of *EGFP* in CDG fibroblasts had no effect on the pathologic profile characterized by the accumulation of DolPP-$GlcNAc_2Man_5$. The retention times of DolPP-$GlcNAc_2Man_1$ (M_1) to DolPP-$GlcNAc_2Man_9Glc_3$ (M_9G_3) are marked at the top of the profiles.

It is noteworthy that a deficiency of MPDU1 in humans leads to CDG, which is associated with the parallel accumulation of the LLO DolPP-$GlcNAc_2Man_5$ and DolPP-$GlcNAc_2Man_9$ (Kranz, et al., 2001; Schenk, et al., 2001b).

To date, 21 forms of CDG have been divided into 13 types of CDG-I and 8 types of CDG-II based on the nature of the glycosylation defects (Aebi, et al., 1999; Freeze, 2006; Jaeken and Matthijs, 2007).

According to the nomenclature guidelines established previously (Aebi, et al., 1999) , we propose to name the RFT1 deficiency CDG-In.

Acknowledgements

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Web Resources

Accession numbers and URLs for data represented in this study are as follows:

GenBank, http://www.ncbi.nlm.nih.gov/Genbank/ for *Homo sapiens RFT1* at the genomic level (accession number NC_00003) and the coding region (accession number BC043595); for *Mus musculus RFT1* the coding region (accession number BC116368); for *Danio rerio RFT1* the coding region (accession number XM_683262); for *Caenorhabditis elegans RFT1* the coding region (accession number NM_001028439); for *Saccharomyces cerevisiae RFT1* the coding region (accession number U15087)

Online Mendelian Inheritance in Man (OMIM), http://www.ncbi.nlm.nih.gov/Omim/ for CDG

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2 RFT1 deficiency in three novel CDG patients

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Contribution:

Mutation analysis

Lentiviral complementation

RFT1 deficiency in three novel CDG patients

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The medical significance of N-glycosylation is underlined by a group of inherited human disorders called Congenital Disorders of Glycosylation (CDG). One key step in the biosynthesis of the $Glc_3Man_9GlcNAc_2$-PP-dolichol precursor, essential for N-glycosylation, is the translocation of $Man_5GlcNAc_2$-PP-dolichol across the endo-plasmic reticulum membrane. This step is facilitated by the RFT1 protein. Recently, the first RFT1-deficient CDG (RFT1-CDG) patient was identified and presented a severe N-glycosylation disorder. In the present study, we describe three novel CDG patients with an RFT1 deficiency. The first patient was homozygous for the earlier reported *RFT1* missense mutation (c.199C>T; p.R67C), while the two other patients were homozygous for the missense mutation c.454A>G (p.K152E) and c.892G>A (p.E298K), respectively. The pathogenic character of the novel mutations was illustrated by the accumulation of $Man_5GlcNAc_2$-PP-dolichol and by reduced recombinant DNase 1 secretion. Both the glycosylation pattern and recombinant DNase 1 secretion could be normalized by expression of normal *RFT1* cDNA in the patients' fibroblasts. The clinical phenotype of these patients comprised typical CDG symptoms in addition to sensorineural deafness, rarely reported in CDG patients. The identification of additional RFT1-deficient patients allowed to delineate the main clinical picture of RFT1-CDG and confirmed the crucial role of RFT1 in $Man_5GlcNAc_2$-PP-dolichol translocation.

Key Words

Glycosylation, Congenital Disorders of Glycosylation, RFT1, dolichol cycle

Introduction

N-glycosylation is a post-translational modify-cation of proteins found in eukaryotic and prokaryotic organisms (Weerapana and Imperiali, 2006). In eukaryotes, protein N-linked glycans are involved in many essential biological processes including the immune response, intracellular targeting, cell-cell recognition, protein folding and protein stability (Varki, 1993).

The eukaryotic N-glycosylation pathway starts with the assembly of the $Glc_3Man_9GlcNAc_2$ oligosaccharide precursor on a dolichol-PP carrier in a well-ordered process known as the dolichol cycle (Burda and Aebi, 1999). This dolichol cycle starts with the elongation of dolichol-P to $Man_5GlcNAc_2$-PP-dolichol on the cytosolic face of the endoplasmic reticulum (ER) membrane, while the elongation of $Man_5GlcNAc_2$-PP-dolichol to the complete $Glc_3Man_9GlcNAc_2$-PP-dolichol occurs in the ER lumen. For this reason, the $Man_5GlcNAc_2$-PP-dolichol intermediate has to be translocated across the ER membrane. In yeast, the ER membrane protein Rft1 was shown to facilitate the translocation of $Man_5GlcNAc_2$-PP-dolichol to the ER lumen in a bidirectional and ATP-independent manner (Helenius, et al., 2002). Once the fully assembled oligosaccharide precursor is synthesized, $Glc_3Man_9GlcNAc_2$ is transferred onto selected asparagine residues of polypeptide chains by the oligosaccharyltransferase (OST) complex (Burda and Aebi, 1999).

Congenital Disorders of Glycosylation (CDG) are a group of inherited human disorders characterized by deficient protein glycosylation. Up to now, 14 different CDG types deficient in protein N-glycosylation site occupancy have been identified. The analysis of the serum sialotransferrin pattern is the most widely used method to screen for N-glycosylation disorders, which can be classified into two subgroups: defects of oligosaccharide

precursor assembly and transfer to proteins (formerly known as CDG-I) and defects of N-glycoprotein processing (formerly known as CDG-II) (Eklund and Freeze, 2006; Leroy, 2006; Freeze, 2007; Jaeken and Matthijs, 2007). Recently, the first RFT1-deficient patient (OMIM 612015) was identified (Haeuptle, et al., 2008). Haeuptle and co-workers described a young girl presenting with marked psychomotor retardation, hypotonia, seizures, hepatomegaly and coagulopathy. This patient was homozygous for the missense mutation c.199C>T (p.R67C) in the *RFT1* gene and accumulated $Man_5GlcNAc_2$-PP-dolichol as shown by lipid-linked oligosaccharides (LLO) analysis. However, no $Man_5GlcNAc_2$ was transferred onto glycoproteins, which pointed to a deficient translocation of the accumulated LLO across the ER membrane.

In the present study, we describe three additional RFT1-deficient patients including two novel pathogenic point mutations. The identification of these additional patients allowed us to refine the clinical phenotype characteristic for RFT1 deficiency, designated as RFT1-CDG according to the suggested novel nomenclature (Jaeken, et al., 2008).

Materials and methods

Cell culture - Primary skin fibroblasts from healthy controls and patients were cultured at 37°C under 5% CO_2 in DMEM/F12 (Life Technologies) supplemented with 10% fetal bovine serum (Clone III, HyClones). Research on patients' cells was prospectively reviewed and approved by the Ethics Committee of the University of Leuven.

Mutation analysis - Total RNA was isolated from 2 x 10^7 fibroblasts using the TRIzol LS reagent (Invitrogen) according to the manufacturer's instructions. The human *RFT1* cDNA was prepared and the protein-coding region was amplified by PCR as described before (Haeuptle, et al., 2008). The PCR products were sequenced (Synergene Biotech) after removal of the unincorporated nucleotides with QIAquick columns (QIAGEN). Carrier analysis in the parents and healthy siblings was performed on DNA extracted from blood. Primers were designed to amplify exons 3, 4 and 9 of the *RFT1* gene, based on the genomic sequence (NM_052859.2). Primer sequences are available on request. These exons were amplified using standard PCR conditions, subsequently sequenced with the Big Dye Terminator Ready reaction cycle sequencing kit V3.1 (Applera) and analyzed on an ABI3100 Avant (Applera). The numbering of the nucleotide changes is based on the cDNA sequence (NM_052859.2) with +1 corresponding to the A of the ATG translation initiation codon in the reference sequence.

Metabolic radiolabelling - Fibroblasts (8 x 10^6 per labelling) were grown overnight in a 175 cm^2 tissue culture flask. After 24 h, cells were pre-incubated in 0.5 mM glucose for 45 min and then pulse radiolabelled for 1 h with 150 μCi of 2-[^{3}H]-mannose (16 Ci/mmol, Amersham Biosciences). After metabolic labelling, the cells were scraped with 1.1 ml MeOH/H_2O (8:3) followed by the addition of 1.2 ml $CHCl_3$. Sequential extraction of oligosaccharide material was performed as previously described (Cacan and Verbert, 1997).

Analysis of oligosaccharide material - Glycoprotein fractions obtained at the end of the sequential extraction were digested overnight at room temperature with trypsin (1 mg/ml; Sigma) in 0.1 M ammonium bicarbonate buffer, pH 7.9. The resulting glycopeptides were treated with 0.5 U PNGase F (Roche) in 50 mM phosphate buffer, pH 7.2 for 4 h to release the oligosaccharides from the peptides. The oligosaccharides were desalted on Bio-Gel P2 columns and eluted with 5% acetic acid. LLO fractions obtained after sequential

extraction were subjected to mild acid treatment (0.1 M HCl in THF) for 2 h at 50 °C. Purification of the released oligosaccharides was performed as described above.The oligosaccharides were separated by HPLC on an amino derivated Asahipak NH_2P-50 column (250 mm x 4.6 mm; Asahi) applying a gradient of acetonitrile/H_2O ranging from 70:30 to 50:50 over 90 min at a flow rate of 1 ml/min. Oligosaccharides were identified on the basis of their retention times compared to standard glycans (Foulquier, et al., 2002). Elution of the radiolabelled oligosaccharides was monitored by continuous β-counting with a flo-one β detector (Packard).

Complementation of the LLO profile – Lenti-viruses containing *RFT1* and *EGFP* cDNAs were prepared as previously described (Haeuptle, et al., 2008). Briefly, 3 x 10^6 HEK293T cells were transfected with 20 µg pLenti6-*hRFT1* or pLenti6-*EGFP* and 36 µg of the packing plasmid mix (Invitrogen) by calcium-phosphate precipitation. Two days after transfection, the supernatants were collected and used to transduce the patient fibroblasts. After selection with 5 µg/ml blasticidin (Invitrogen), the cells were metabolically labelled with 2-[^{3}H]-mannose and the extracted LLO were analysed by HPLC (Zufferey, et al., 1995).

Complementation of DNase 1 secretion - Bovine *DNase I* cDNA was subcloned into the pSVK3 vector as previously reported (Nishikawa, et al., 1997). Patients' fibroblasts were transduced with an adenovirus expressing bovine *DNase I* (Eklund, et al., 2005a; Fujita, et al., 2008). Two days post transduction, the cells were washed twice with PBS and incubated at 37 °C with 1 ml DMEM without methionine/cysteine, containing 10% dialysed fetal bovine serum, 10 mM NH_4Cl and 0.2 mCi [^{35}S]-Met/Cys labelling mixture. After a labelling time of 4 h, the culture medium was harvested and DNase 1 was immunoprecipitated as previously described (Nishikawa and Mizuno, 2001). The immunoprecipitated samples were subjected to SDS-PAGE using a 13% acrylamide gel. After electrophoresis, the gels were rinsed with a mixture of 7% acetic acid and 10% methanol for 15 min and soaked in Amplify solution (Amersham Pharmacia Biotech) for 15 min. The gels were dried, autoradiography was carried out and the intensity of the bands corresponding to DNase I was quantified by scanning densitometery (using Quantity One software PDI). Band intensity was normalized against protein levels.

Results

Biochemical and molecular diagnosis

Three patients with a type 1 pattern of serum sialotransferrins were further investigated. Subsequently, phosphomannomutase (*CDG-Ia*) and phosphomannose isomerase (*CDG-Ib*) deficiencies were excluded on the basis of enzymatic activity measurements in the patients' fibroblasts (data not shown).

In order to identify a defect in the assembly of the oligosaccharide precursor within the dolichol cycle, structural analysis of the LLO was performed by HPLC after metabolic labelling. In healthy control fibroblasts, the LLO profile is characterized by the fully assembled oligosaccharide precursor $Glc_3Man_9GlcNAc_2$-PP-dolichol. In contrast, an accumulation of $Man_5GlcNAc_2$-PP-dolichol in combination with only a minor amount of complete LLO was detected in all three patients (Figure 1, panels A, B, C and D). Additionally, protein-linked glycan structures were analyzed. In both, healthy control and patients, $Man_8GlcNAc_2$, $Man_9GlcNAc_2$ and $Glc_1Man_9GlcNAc_2$ structures were obtained

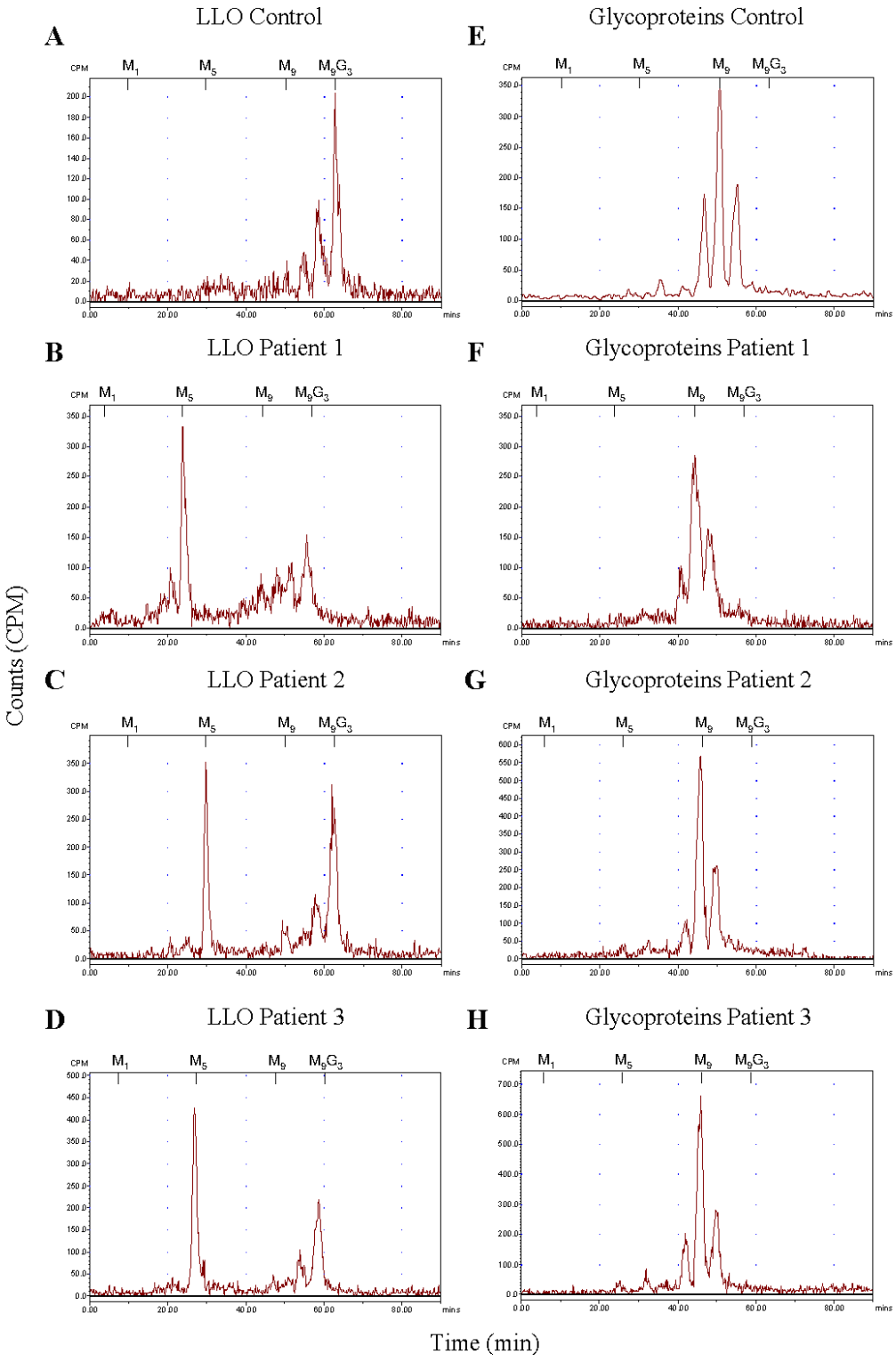

Figure 1: (A-D) HPLC analysis of the LLO in control and patients' fibroblasts revealing the accumulation of $Man_5GlcNAc_2$-PP-dolichol in the three patients. **(E-H)** Protein N-linked oligosaccharides of control and patients were separated by HPLC, demonstrating that no aberrant glycan structures were detected in the patients. The retention times of the standard oligosaccharides $Glc_3Man_9GlcNAc_2$-PP-dolichol (G_3M_9) and $Man_{1\text{-}9}GlcNAc_2$-PP-dolichol ($M_{1\text{-}9}$) are marked above the HPLC profiles.

(Figure 1, panels E, F, G and H). Notably, no $Man_5GlcNAc_2$ structure could be detected on the patients' glycoproteins.

The accumulation of $Man_5GlcNAc_2$-PP-dolichol on LLO combined with normal protein-linked glycans is typical for a defect in the translocation of $Man_5GlcNAc_2$-PP-dolichol to the ER lumen and was recently reported in a RFT1-deficient patient (OMIM 612015) (Haeuptle, et al., 2008). To this end, mutation analysis of the *RFT1* cDNA was performed in the present patients and all three carried RFT1 mutations. The first patient was homozygous for the earlier reported missense mutation (c.199C>T; p.R67C) (Haeuptle, et al., 2008), while the parents were heterozygous carriers of this mutation (data reviewed but not shown). Two novel point mutations were identified in two other patients. Sequencing of the second patient's *RFT1* cDNA revealed an A to G transition at nucleotide position 454, leading to the conversion of a lysine into a glutamic acid at position 152 on the protein level (data reviewed but not shown). Both parents as well as a healthy sibling were heterozygous for this mutation. In model eukaryotic organisms, the lysine at position 152 is mostly conserved and a conversion of a lysine into a glutamic acid was not observed (data not shown). The third patient was homozygous for a G to A transition at nucleotide position 892, causing a glutamic acid to lysine change at position 298 (data reviewed but not shown). The mutated glutamic residue is strictly conserved among eukaryotes (data not shown). No material of the parents and siblings was available for carrier analysis.

In addition, all converted amino acids were found in hydrophilic domains of the RFT1 protein predicted to be oriented to the ER lumen (TMpred (Hofmann and Stoffel, 1993); TMHMM (Krogh, et al., 2001)) (Figure 2). As previously reported, the p.R67C mutation is located in the first luminal loop of the RFT1 protein (Haeuptle, et al., 2008). The p.K152E mutation was found in the second luminally oriented 25 amino acid long hydrophilic stretch and the p.E298K mutation was positioned in the largest luminal loop, bearing a potential N-glycosylation site at position p.N227 and ranging over 130 amino acids (Figure 2).

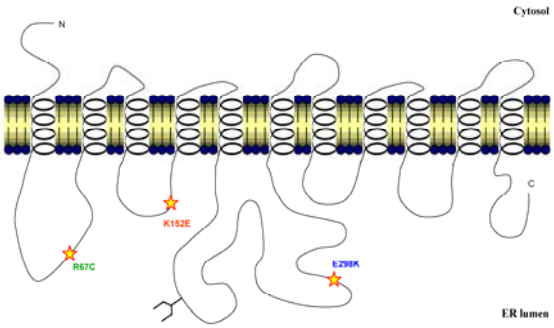

Figure 2: Schematic representation of the identified missense mutations in the predicted topology of the RFT1 protein. All three mutations were found in the predicted luminal loops of the protein. The orientation of the model was supported by the favoured position of the 11 transmembrane domains and the localization of a potential N-glycosylation site (p.N227) (TMpred (Hofmann and Stoffel, 1993); TMHMM (Krogh, et al., 2001)).

Complementation of the LLO profile

To demonstrate the pathogenicity of the two new missense mutations, wild-type *RFT1* cDNA was transduced into patients' fibroblasts using a lentiviral construct to complement the $Man_5GlcNAc_2$-PP-dolichol accumulation. Healthy control and patients' fibroblasts were thus infected with recombinant lentiviruses expressing either the wild-type *RFT1* cDNA or *EGFP* as a negative control. Compared to *EGFP* expression in the patients' fibroblasts, analysis after *RFT1* expression revealed a normalization of the LLO profile characterized by decreased levels of

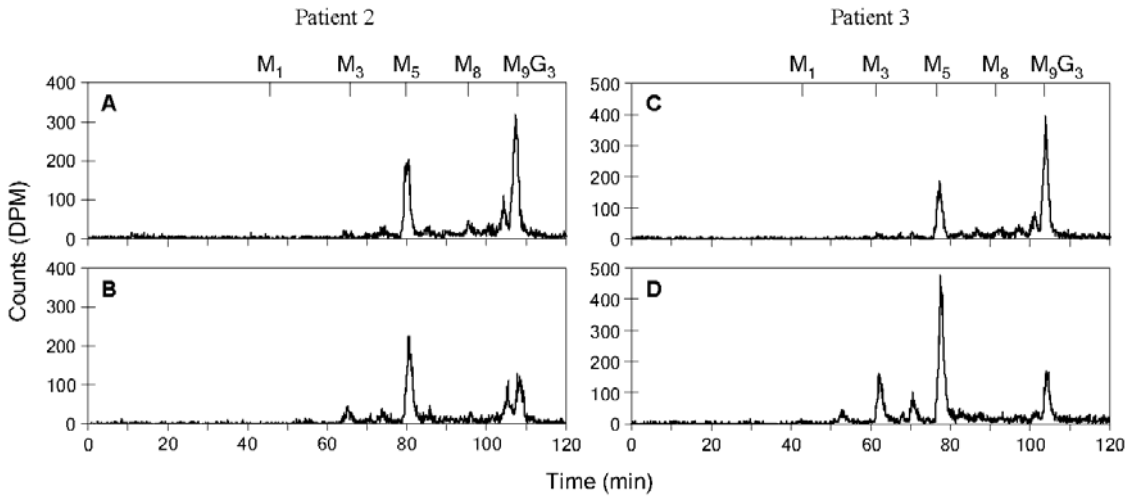

Figure 3: Complementation of the $Man_5GlcNAc_2$-PP-dolichol accumulation. Fibroblasts of patient 2 **(A)** and patient 3 **(C)** expressing wild-type *RFT1* cDNA, leading to a decreased accumulation of $Man_5GlcNAc_2$-PP-dolichol. Lentiviral mediated expression of *EGFP* in the fibroblasts of patient 2 **(B)** and patient 3 **(D)** showed no effect. The retention times of the standard oligosaccharides $Glc_3Man_9GlcNAc_2$-PP-dolichol (G_3M_9) and $Man_{1\text{-}9}GlcNAc_2$-PP-dolichol ($M_{1\text{-}9}$) are marked above the HPLC profiles.

$Man_5GlcNAc_2$-PP-dolichol and increased levels of the complete $Glc_3Man_9GlcNAc_2$-PP-dolichol (Figure 3). *EGFP* expression in the patients' fibroblasts did not affect the LLO profile, as expected.

Complementation of DNase 1 secretion

In another alternative method to prove the pathogenicity of the two new mutations, secretion of recombinant bovine DNase 1 was investigated in all three RFT1-deficient patients. A modified version of bovine DNase 1 has a single potential N-glycosylation site (p.N106) and is secreted when expressed in human fibroblasts (Nishikawa and Mizuno, 2001). However, upon expression in the fibroblasts of CDG patients, DNase 1 secretion was strongly reduced (Eklund, et al., 2005a; Fujita, et al., 2008). As shown in Figure 4 (lanes 3, 5 and 7), DNase 1 secretion was significantly reduced in all three RFT1-deficient patients. Transduction of the patients' fibroblasts with lentiviruses coding for wild-type *RFT1* restored the levels of DNase 1 secretion (Figure 4, lanes 4, 6 and 8), thus three different patients were compared. Patient 1 was the first child of healthy, unrelated North Americans of Scottish-English origin and presented with respiratory insufficiency, severe generalized epilepsy with intractable seizures, infantile spasms, microcephaly, failure to thrive, hypotonia, sensorineural deafness and decreased visual acuity. In addition, this patient showed severe mental retardation, with virtually no development, and had normal liver function. Dysmorphic features included micrognathia, short neck, small nose, drooping eyelids, valgus feet and adducted thumbs. This patient also had severe feeding problems requiring gastrostomy and died at the age of eight months. Brain MRI at early age did not reveal cerebral nor cerebellar atrophy. However, at autopsy, weight and gyral/sulcal pattern consistent with a degree of cerebral atrophy was reported (Table 1).

Patient 2 was the second son of healthy, consanguineous Italian parents. During the first year of life, he presented with severe developmental delay, microcephaly, nystagmus,

		Patient 1	Patient 2	Patient 3	Patient KS
Gender		female	male	male	female
Origin		Scottish	Italian	Algerian	English
Vital status		died 8 m	alive 5.5 y	alive 2.2y	died 4.3 y
Consanguinity		-	+	+	-
Feeding problems		+	+	+	+
Failure to thrive		+	+	+	+
Severe mental retardation		+	+	+	+
Microcephaly		+	+	-	na
Hypotonia		+	+	+	+
Epilepsy		+	+	+	+
Decreased visual acuity		+	+	+	+
Sensorineural deafness		+	+	+	+
Myoclonic jerks		+	+	+	+
Respiratory insufficiency		+	-	+	na
Pulmonary infections		-	+	+	+
Coagulopathy		na	+	na	+
Dysmophic features:	micrognathia	+	-	+	na
	short neck	+	-	+	na
	valgus feet	+	+	+	na
	adducted thumbs	+	na	+	-
	kyphoscoliosis	na	+	na	+
	inverted nipples	-	+	+	+
Brain MRI:	cerebral atrophy	-*	+	-	+
	cerebellar atrophy	-	-	-	+

Table 1: Clinical phenotype of the three new RFT1-deficient patients compared to the phenotype of the originally reported case (KS) (Imtiaz, et al., 2000; Haeuptle, et al., 2008; Clayton and Grunewald, 2009).
Symbols: na: not available; *: cerebral atrophy reported at autopsy.

sensorineural deafness, relapsing aspiration pneumonia, a generalized hypotonia and inverted nipples. Additionally, this patient was frequently hospitalized because of drug-resistant epilepsy and feeding problems requiring gastrostomy. At the age of 4 years, computer tomography scan of the brain revealed a stroke-like episode affecting the left frontal lobe, and the following year he started to suffer from recurrent deep venous thrombosis of the left leg. Clinical examination at the age of five years showed a severe mentally retarded child with microcephaly, spastic tetraparesis and kyphoscoliosis. Serial brain MRI examinations showed progressive cortical and subcortical atrophy with no cerebellar involvement. Liver function was normal (Table 1).

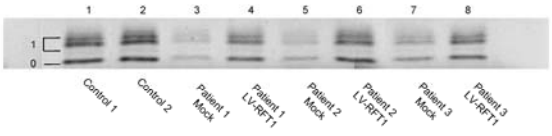

Figure 4: Rescue of DNase 1 secretion in the patients' fibroblasts. Secretion of DNase 1 is reduced in the fibroblasts of the three RFT1-deficient patients (lanes 3, 5 and 7). Expression of wild-type *RFT1* cDNA (lanes 4, 6 and 8) restored the secretion of DNase 1 to a level equal to controls (lanes 1 and 2). Symbols: o = non-glycosylated DNase 1; 1 = singly glycosylated DNase 1.

The third patient was the ninth child of healthy, consanguineous parents from Algerian origin. This family has two children presenting with the autosomal recessive disorder *Hemophagocytic Lymphohistiocytosis* (OMIM 267700). One child died at the age of three months, while the other is still alive due to bone marrow transplantation at the age of four months. In addition, one son died at the age of four days and presented with similar characteristics as patient 3. Patient 3 presented with respiratory insufficiency, hypotonia, body spasm, failure to thrive, epilepsy, bilateral glaucoma and sensorineural deafness. Dysmophic features included slightly inverted nipples, infiltrated ears, short neck, retrognathism, glossoptosis, adducted thumbs and valgus feet. Brain MRI did not show cerebral or cerebellar atrophy. This patient also had feeding problems and chronic infections of the respiratory tract. In addition, he showed severe mental retardation with the absence of visual contact. Liver function was normal (Table 1).

Discussion

Based on the analysis of LLO and protein-linked glycan profiles in fibroblasts, three potential RFT1-deficient patients were identified. All three patients showed an accumulation of the LLO $Man_5GlcNAc_2$-PP-dolichol, while no $Man_5GlcNAc_2$ was detected on glycoproteins. Mutation analysis of the *RFT1* gene revealed that one patient was homozygous for the earlier reported missense mutation (c.199C>T, p.R67C). This is the second report of the p.R67C mutation in a patient of British origin (Imtiaz, et al., 2000) and there are no indications that both patients are related. This could thus point to a founder effect of the p.R67C mutation. The other patients were homozygous for the new missense mutations c.454A>G (p.K152E) and c.892G>A (p.E298K). The pathogenic character of these novel mutations was demonstrated by the complementation of the abnormal LLO profile and reduced DNase 1 secretion upon expression of wild-type *RFT1* in the patients' fibroblasts.

All three *RFT1* mutations identified so far are located in one of the hydrophilic loops predicted to be within the ER lumen. It can be assumed that these regions are of major importance for the translocation of $Man_5GlcNAc_2$-PP-dolichol in the ER lumen or for the maintenance of $Man_5GlcNAc_2$-PP-dolichol on the luminal side. Further structural analysis will be required to confirm the predicted orientation of the RFT1 protein. Determination of the occupancy of the putative N-glycosylation site at position p.N227 (Figure 2) would certainly contribute to establish the topology of the RFT1 protein.

In yeast, the Rft1 protein was genetically identified as a protein mediating the translocation of LLO across the ER membrane (Helenius, et al., 2002). However, recent evidence suggests that the RFT1 protein would not be the flippase enzyme itself, but would play a critical accessory role in translocating $Man_5GlcNAc_2$-PP-dolichol to the ER lumen (Frank, et al., 2008; Sanyal, et al., 2008). Anyhow, the identification of three additional RFT1-deficient patients clearly underscores the

major importance of RFT1 in this translocation event.

Finally, the identification of three additional patients allowed us to refine the clinical phenotype characteristic for RFT1 deficiency (OMIM 612015). All four known RFT1-deficient patients showed very similar characteristics including severe mental retardation, hypotonia, epilepsy, myoclonic jerks, decreased visual acuity, sensorineural deafness and feeding problems (Table 1). In comparison to other CDG defects, RFT1 deficiency is thus mainly a neurological disorder. Strikingly, sensorineural deafness was found in all four RFT1-deficient patients (Imtiaz, et al., 2000) and might represent a characteristic clinical feature of RFT1-CDG, since deafness has been reported in only a few other CDG patients (Hutchesson, et al., 1995; Imtiaz, et al., 2000; Kranz, et al., 2007a).

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3 A new case of ALG8 deficiency (CDG Ih)

(Journal of Inherited Metabolic Disease. 2009; *in press*)

Contribution:

LLO analysis

NLO analysis

A new case of ALG8 deficiency (CDG-Ih)

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Congenital disorders of glycosylation (CDG) represent an expanding group of inherited diseases. One of them, ALG8 deficiency (CDG-Ih), leads to protein N-glycosylation defects caused by malfunction of glucosyltransferase 2 (Dol-P-Glc: Glc1-Man$_9$-GlcNAc$_2$-P-P-Dol-glucosyltransferase) resulting in inefficient addition of the second glucose residue onto lipid-linked oligosaccharides. So far, only five patients have been described with ALG8 deficiency. We present a new patient with neonatal onset. The girl was born at the 29th week of gestation complicated by oligo-hydramnios. Althoug the early postnatal adaptation was uneventful (Apgar score 8 and 9 at 5 and 10 min), generalized oedema, multifocal myoclonic seizures, and bleeding due to combined coagulopathy were present from the first day. Diarrhoea progressing to protein-losing enteropathy with ascites and pericardial effusion developed in the third week of life. Pharmacoresistent seizures and cortical, cerebellar and optic nerve atrophy indicated neurological involvement. No symptoms of liver disease except coagulopathy were observed; however, steatofibrosis with cholestasis was found at autopsy. The girl died at the age of 2 months owing to the progressive general oedema, bleeding and cardio-respiratory insufficiency. Molecular analysis revealed two heterozygous mutations in the *ALG8* gene: c.139A>C (p.T47P) and the novel mutation c.1090C>T (p.R364X). *Conclusion:* The prognosis of patients with ALG8 deficiency is unfavorable. The majority of affected children have early onset of the disease with heterogeneous symptoms including multiple organ dysfunction, coagulopathy and protein-losing enteropathy. Neurological impairment is not a general clinical symptom, but it has to be taken into consideration when thinking about ALG8 deficiency.

Abbreviations

CDG, congenital disorders of glycosylation; CDT, carbohydrate-deficient transferrin; Dol, dolichol; IEF, isoelectric focusing; LLO, lipid-linked oligosaccharide; NLO, protein N-linked oligosaccharide; PLE, protein-losing enteropathy; PMI phosphomannose isomerase; PMM phosphomannomutase

Introduction

Congenital disorders of glycosylation (CDG) comprise a large group of genetic diseases resulting from defects in the synthesis and the processing of glycans (Jaeken and Matthijs, 2007). ALG8 deficiency (CDG-Ih; OMIM 608104) is caused by pathological mutations in the *ALG8* gene (Chantret, et al., 2003) coding for the enzyme glucosyltransferase 2 (Dol– P- Glc:Glc1- Man$_9$-GlcNAc$_2$ –P –P -Dol glucosyltransferase). This enzyme is responsible for the addition of the second glucose residue onto the growing lipid-linked oligosaccharide (LLO) chain (Chantret, et al., 2003). The *ALG8* gene is located on chromosome 11 and spans 38.7 kbp. Translation of its 1404-nucleotide-long open reading frame, organized in 13 exons, results in a 467-amino-acid product.

So far, only five patients with ALG8 deficiency have been described (Charlwood, et al., 1997; Chantret, et al., 2003; Schollen, et al., 2004; Eklund, et al., 2005c), presenting with dysmorphic features, hypotonia, oedema, coagulopathy, gastrointestinal disorders including protein-losing enteropathy (PLE), hepatomegaly and cardiorespiratory problems. Four patients had neonatal onset and deceased in early childhood. One patient (Chantret, et al., 2003) manifested

later at the age of 4 month and survived beyond 3 years of age.

The aim of our study was to present a detailed clinical picture of a new ALG8-deficient patient with neonatal onset to further expand knowledge of the clinical symptoms in this disorder.

Case report

The girl was the first child of healthy, unrelated caucasian parents. The pregnancy after *in vitro* fertilization was complicated with oligo-hydramnios progressing to anhydramnios. The child was born at the 29th week of gestation with a birth weight of 1420 g and a length of 38 cm by acute Caesarean section. Generalized oedema (anasarca) and mild hypotonia were observed from birth. Although, the early postnatal adaptation was viable, with Apgar score 8 and 9 at 5 and 10 min, respectively, multifocal myoclonic seizures and clinical signs of severe anaemia developed during the first day of life. She was ventilated and inotropic treatment was initiated. Each attempt at sucking from an endotracheal tube was complicated by mild bleeding episodes. Laboratory analyses at that time revealed pancytopenia (leukocytes 2.6x10^9/L, controls >5.5; haemoglobin 85 g/L, controls >145; thrombocytopenia 61x10^9/L, controls >150) and severe combined coagulopathy with very low levels of factor XI (2% in comparison with controls), antithrombin III (20%) and protein C (2%). Activated partial thromboplastin time (APTT) was markedly prolonged (>180s). Almost no improvement of the coagulation status was seen after frequent administration of fresh frozen plasma, antithrombin III, protein C and factor VIIa concentrate. Haemorrhagic diathesis with bleeding necessitated repeated administration of packed red blood cells.

Although the clinical status appeared to be stabilized, from the third week of life progressive diarrhoea, intolerance of nasogastric tube feeding, abdominal distension and subsequent development of ascites and pericardial effusion were observed, as well as hypoalbuminaemia (21.6 g/L, controls >35 g/L). Bleeding predominated over tendency to thrombosis, but thrombosis of the vena cava inferior was documented at the age of 1 month. As a consequence of cardiac insufficiency and thrombotic episodes, liver congestion and portal hypertension developed, leading to marked hepatospenomegaly.

Oedema was permanent with progressive course. Even though we did not study α_1-antitrypsin clearance, the diagnosis of PLE was highly probable on the basis that maintenance of the normal concentration of albumin and immunoglobulins in blood necessitated practically continuous infusion of albumin (10% solution) and immunoglobulin substitution and that diarrhoea persisted and loss of albumin into the urine was minimal.

The seizures were under control on phenobarbital treatment, but generalized tonic seizures appeared at the age of 6 weeks. Brain ultrasound and eye investigations did not show any pathological findings until the age of 6 weeks, when mild cerebral cortical atrophy and bilateral optic nerve atrophy were observed.

The girl was oxygen-dependent on artificial lung ventilation or nasal continuous positive airway pressure until her death at the age of 2 months. The steatofibrosis with cholestasis found at autopsy was striking, since no laboratory signs of liver failure or marked liver dysfunction were observed during the patient's life. Brain postmortem studies revealed mild cerebellar atrophy, but showed no specific signs of cortical

dysgenesis including the changes in the periventricular area typical for premature newborns.

The immediate cause of death was the progressive general oedema, bleeding and cardio-respiratory insufficiency leading to multiple organ failure.

Ethics

The study was carried out in accordance with the Declaration of Helsinki of the World Medical Association and was approved by the Committee of Medical Ethics of the Faculty of Medicine and General Teaching Hospital in Prague. Informed consent was obtained from the parents.

Methods

Biochemical methods

Isoelectric focusin (IEF) of serum transferrin was carried out as described by van Eijk and van Noort (van Eijk and van Noort, 1992). Iron-saturated serum was incubated overnight at 37°C with neuraminidase from *Clostridium perfringens* (Boehringer-Ingelheim, Praha, Czech Republic), and subsequently separated by SDS-PAGE (5%T, 3%C; pH 3-10). Phosphomannomutase (PMM) and phosphomannose isomerase (PMI) activities were measured in isolated lymphocytes according to the procedure described by Van Schaftingen and Jaeken (Van Schaftingen and Jaeken, 1995).

LLOs and protein N-linked oligosaccharides (NLOs) were prepared from control and patient fibroblasts as described previously (Haeuptle, et al., 2008). Briefly, the oligosaccharides were metabolically labelled by incorporation of [^{3}H]-mannose. The LLOs were extracted with organic solvents and hydrolysed by mild acid treatment. NLOs were released from glycoproteins by incubation with PNGase F (New England BioLabs, Ipswich, USA). Prior to HPLC analysis, the oligosaccharides were purified by ionic and hydrophobic chromatography.

Molecular methods

All 13 exons of the *ALG8* gene amplified from patient's gDNA were analyzed on an ABI PRISM 3100-Avant Genetic Analyzer (Applied Biosystems, Foster City, USA). The c.139A>C mutation was confirmed by restriction fragment length polymorphism using the *Mnl*I restriction endonuclease. For confirmation of the c.1090C>T mutation, we designed mismatch primers for the *Hpa*II enzyme.

Results

Biochemical analyses

The possible diagnosis of CDG in this patient was confirmed by detection of hypoglycosylated transferrin isoforms by IEF. The analysis revealed an abnormal glycosylation pattern, with decreased tetrasialo- and increased disialo- and asialo-transferrin, which is typical for CDG type I. Neuraminidase treatment excluded the possibility of a transferrin polymorphism. PMM2 and PMI activities measured in isolated lymphocytes and cultivated fibroblasts were normal, thus excluding CDG-Ia and CDG-Ib.

LLO analysis

In order to detect a possible defect in LLO assembly, skin fibroblasts isolated from the patient were labeled with [^{3}H]-mannose, and the glycans released from the lipid carrier were analyzed by HPLC. The LLO profile of the patient showed the accumulation of incomplete precursor structures corresponding to $GlcNAc_2Man_9$ and $GlcNAc_2Man_9Glc_1$ (Fig. 1, left panel). This pattern is typical for ALG8 deficiency (CDG-Ih) (Chantret, et al., 2003).

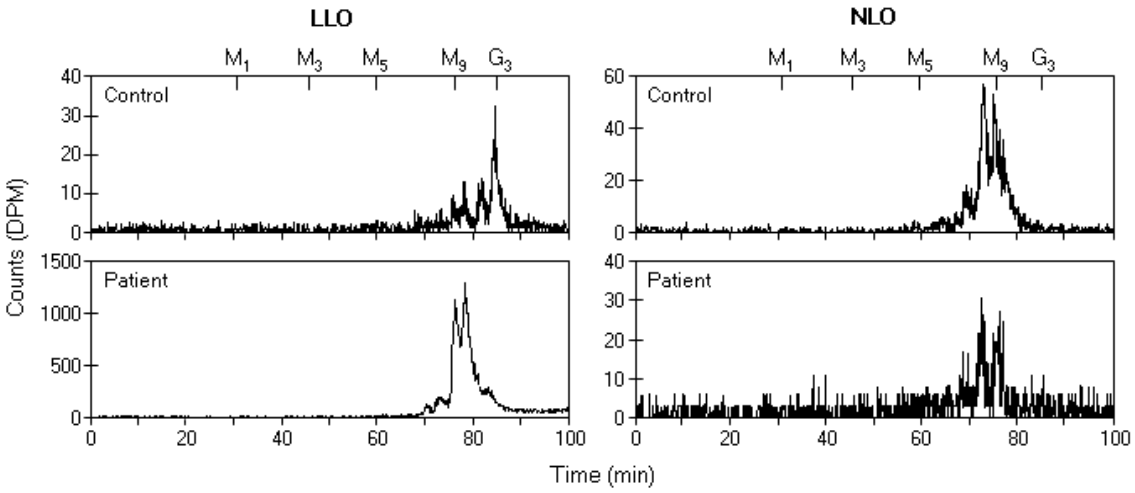

Figure 1. Analysis of lipid- and N-linked oligosaccharides in patient and control fibroblasts. Oligosaccharides from control (upper panels) and patient cells (lower panels) were metabolically labeled with [^{3}H]-mannose. LLO were extracted, purified and analyzed by HPLC. The elution profiles (left panels) show the accumulation of the intermediate Dol-PP-$GlcNAc_2Man_9$ and -$GlcNAc_2Man_9Glc_1$ in the patient's cells. Enzymatically released NLO from control and patient fibroblasts yielded qualitatively similar profiles (right panels). The retention times of Dol-PP-$GlcNAc_2Man_{1\text{-}9}$ ($M_{1\text{-}9}$) and -$GlcNAc_2Man_9Glc_3$ (G_3) are indicated at the top of panels.

The accumulation of Dol-PP-$GlcNAc_2Man_9$ observed in the patient may be due to the deglucosylation of Dol-PP-$GlcNAc_2Man_9Glc_1$ by ER glucosidase II that has arisen owing to inefficient addition of the second glucose residue by the ALG8 glucosyltransferase.

NLO analysis

The NLO analysis revealed an HPLC profile comparable to that obtained from healthy control subjects (Fig. 1, right panel). This is in accordance with thin-layer chromatography analysis of NLO in an earlier ALG8-deficient patient (Chantret, et al., 2003).

Molecular analyses

Two *ALG8* mutations in heterozygous form were detected in the patient. The first mutation (c.139A>C), already described in the literature (Schollen, et al., 2004), was combined with a novel mutation c.1090C>T. The index mutation, which is translated into p.T47P, was inherited from the father. The c.1090C>T mutation resulting in a premature stop codon (p.R364X) was found in heterozygous form in the mother, whereas it was not found in 150 healthy controls.

Discussion

The clinical course of the disease in our patient demonstrates, similarly to the published cases (see Table 1), that the principal clinical and laboratory pathology is haemato-intestinal presentation. The majority of the patients were delivered prematurely and the first clinical symptoms, especially PLE, were presented during the first weeks of life. Only in one patient (surviving beyond 3 years of age) did PLE and diarrhoea develop later at the age of 4 months.

Anaemia, pancytopenia, hypoalbuminaemia and coagulopathy were observed in most patients. Coagulopathy in patients with ALG8 deficiency appears to be caused by a combination of hypoproduction of both clotting factors and their

	Chantret et al. (2003)	Schollen et al. (2004) GB0243	GB0244	NL0097	Eklund et al. (2005)	new case (2008)
Sex	F	M	F	M	M	F
Prenatal data						
IUGR	ND	-	+	-	-	-
Oligohydramnion	ND	+	ND	-	-	+
Reduced fetal movement	ND	+	+	-	-	-
Postnatal data						
Week of gestation	ND	35 (CS)	ND	35	37	29 (CS)
Birth weight (g)	ND	2280	ND	ND	3070	1420
Onset of first symptoms	4 months	From birth	From birth	8 weeks	From birth	From birth
Failure to thrive	ND	+	ND	+	ND	+
Dysmorphy	-	+	ND	+	+	-
Cryptorchism		+		+	ND	
Diarrhoea	+	+	ND	-	+	+
Ascites	+	+	+	+	+	+
Pericardial efussion	-	+	ND	-	ND	+
Protein-losing enteropathy	+	+	ND	ND	+	+
Hepatomegaly	+	-	ND	ND	+	+
Hypotonia	-	-	ND	+	+	+
Microcephaly	-	+	ND	-	-	-
Psychomotor delay	-	-	ND	-	+	+
Seizures	-	-	-	-	+	+
Lung hypoplasia	-	-	ND	+	-	-
Eye symptoms	Retinopathy	ND	ND	ND	Cataracts	Optic atrophy
Exitus	Alive?[a]	3 months	3 days	3 months	16 months	2 months
Laboratory data						
Coagulopathy	+	+	ND	ND	+	+
Anaemia	ND	+	ND	+[c]	+[b]	+[c]
Proteinuria	ND	+	ND	ND	+	+
Tubulopathy	ND	+	ND	ND	+	-
Hepatopathy	-	-	ND	ND	+	-
Hypoalbuminaemia	+	+	ND	+	+	+
Elevated CSF protein	ND	ND	ND	ND	+	+
Autopsy						
Cholestasis	ND	ND	ND	+	-	+
Microcystosis of the kidney	ND	ND	ND	+	-	-

Table 1 Overview of clinical and laboratory data in six patients with ALG8 deficiency
IUGR, intrauterine growth retardation; CS, Caesarean section; CSF, cerebrospinal fluid; ND, not described.
[a] Survived beyond 3 years of age [b] Pancytopenia. [c] Thrombocytopenia.

inhibitors, and their increased consumption during chronic disseminated intravascular coagulation. The coagulopathy in our patient seems to be more accentuated with profound bleeding in contrast to patients with other CDG syndromes. Difficult-to-treat hypo-albuminaemia

and coagulopathy resulted in the development of ascites, pericardial effusion, bleeding and early death.

Although craniofacial dysmorphy, inverted nipples and atypical fat pads were not present in our patient, dysmorphy was observed in three reported cases of ALG8 deficiency (Schollen, et al., 2004; Eklund, et al., 2005c), whereas inverted nipples and fat pads were noted in one case (Charlwood, et al., 1997). Since osteopenia, cataracts (Eklund, et al., 2005c), lung hypoplasia (Schollen, et al., 2004) and retinopathy (Chantret, et al., 2003) were found only in a single child, we deduce that these symptoms cannot be considered typical for ALG8-deficient patients.

We did not observe elevated amino-transferases, but pronounced steatofibrosis with cholestasis was found at autopsy. Based on revision of clinical and laboratory reports from all six patients including our case, it seems that mild tubulopathy and proteinuria also belong to typical ALG8 deficiency symptoms. In addition, renal cortical and medullar microcysts were found in our patient.

Until the fifth case was published in 2005 by Eklund and colleagues (Eklund, et al., 2005c), ALG8 deficiency had been presented as a type of CDG without neurological manifestations (Schollen, et al., 2004). Eklund and colleagues described a patient who died at the age of 16 months and presented with psychomotor delay, hypotonia and leukoencephalopathy. In our case, we also observed marked hypotonia and multifocal myoclonic seizures from birth, bilateral optic nerve atrophy, cerebral cortical atrophy and mild cerebellar atrophy. On the other hand, the patient studied by Chantret and co-workers (Chantret, et al., 2003) survived beyond 3 years (Eklund, et al., 2005c) and had no signs of any neurological impairment and, strikingly, PLE and diarrhoea subsequently disappeared. The mild clinical features of this patient could be explained by an elevated residual ALG8 expression. In summary, neurological impairment is not a general clinical symptom, but it has to be taken into consideration when thinking about ALG8 deficiency.

Conclusion

We present a new patient with ALG8 deficiency (CDG Ih), the sixth case described to date. The course of the disease in this new ALG8 case further expands the awareness of the diversity of clinical symptoms, which include multiorgan dysfunction with PLE, liver, kidney and central nervous system involvements and combined coagulopathy. Furthermore, we also identified a novel pathological mutation c.1090C>T (p.R364X) in the *ALG8* gene. The diagnostics enabled genetic counselling and successful prenatal diagnostic in the affected family.

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4 HPLC and mass spectrometry analysis of dolichol-phosphates at the cell culture scale

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Contribution:

Preparation of cellular dolichol-phosphates
9-ADM labelling of dolichol-phosphates
HPLC analysis
Quantification of dolichol-phosphates

HPLC and mass spectrometry analysis of dolichol-phosphates at the cell culture scale

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Dolichols (Dol) are polyprenol lipids that are essential structural components of eukaryotic membranes. In addition, the phosphorylated derivatives of Dol function as lipid anchor of mono- and oligosaccharide precursors involved in protein glycosylation. The biological importance of Dol-phosphates (Dol-P) is illustrated by the severe outcome of human disorders linked to Dol biosynthetic defects, such as Dol-kinase deficiency. For characterization of inherited human diseases and evaluation of therapeutic trials, cultured cells often serve as a sole possible source for experimentation. Limited amounts of cell culture material render the quantitative analysis of Dol a challenging task. Here, we present HPLC and mass spectrometry based approaches to analyse and quantitate Dol-P from cultured human cells. The composition of naturally occurring Dol-P and the saturation state of the α-isoprene units was identified by negative ion electrospray ionization mass spectrometry. Furthermore, fluorescently labelled Dol-P were separated by HPLC and quantified by comparison to known amounts of the internal standard polyprenol-P. The effect of pravastatin, a 3-hydroxy-3-methyl-glutaryl coenzyme-A reductase inhibitor, on the formation of Dol-P in HeLa cells was investigated. As expected, this treatment led to a decrease of Dol-P down to 35% of normal levels.

Keywords

Dolichol-phosphate, mass spectrometry, HPLC, cholesterol pathway, statin, glycosylation

Abbreviations

9-ADM, 9-anthryldiazomethane; Dol, dolichol; Dol-P, dolichol-phosphate; ESI-MS, electrospray ionization mass spectrometry; CDG, Congenital Disorders of Glycosylation

Introductory statement

Dolichols (Dol) are essential components of eukaryotic membranes, where they contribute to the organisation and fluidity of the lipid bilayer and enhance vesicle fusion (Chojnacki and Dallner, 1988). Their composition varies between the kingdoms of life in respect to the number of isoprene subunits, although all Dol are assembled in the identical *trans-trans*-poly*cis* conformation. In contrast to prokaryotic polyprenols, Dol α-isoprene residues are saturated (Swiezewska and Danikiewicz, 2005). Starting from acetate, the pathway leading to the formation of Dol leads via mevalonate to farnesyl-pyrophosphate and is up to this point identical to the cholesterol and ubiquinone biosynthesis pathway (Chojnacki and Dallner, 1988). The enzyme *cis*-prenyltransferase catalyzes the elongation of farnesylpyro-phosphate by sequential condensation of iso-pentenyl-pyrophosphate (Swiezewska and Danikiewicz, 2005). Prior to the NADPH-dependent reduction of the α-isoprene unit, the entirely unsaturated polyprenol-pyrophosphate intermediates are dephosphorylated by mono- or pyro-phosphate phosphatases. In a terminatory step, the resulting Dol are phosphorylated by the CTP-dependent Dol-kinase (Schenk, et al., 2001a).
Dolichol-phosphates (Dol-P) serve as lipid carriers of mono- and oligosaccharides involved in several protein glycosylation pathways (Kornfeld and Kornfeld, 1985; Strahl-Bolsinger, et al., 1999) and in the formation of the glycosylphosphatidyl-inositol anchor (Kinoshita, et al., 1997). Pathogenic alterations of Dol and Dol-P levels and composition have been observed in the context of liver cancer (Eggens, et al., 1988), Alzheimer's disease (Soderberg, et al., 1992), ceroid

lipofuscinosis (Hall and Patrick, 1985) and Dol-kinase deficiency (Kranz, et al., 2007b).

To date, only few techniques have been described for the analysis and quantification of Dol-P (Keller, et al., 1981; Bizri, et al., 1986; Elmberger, et al., 1989). HPLC-based methods separate radio-labelled or fluorescently tagged polyprenol-P and are mainly restricted to tissue-derived samples (Chaudhary, et al., 1982; Yamada, et al., 1986). Whereas several mass spectrometry (MS) methods for the analysis of free Dol have been described (Griffiths, et al., 1996; Skorupinska-Tudek, et al., 2003; D'Alexandri, et al., 2006; Garrett, et al., 2007; Zhang, et al., 2008; Ward, et al., 2009), no MS method has been established to analyse Dol-P from mammalian sources, despite the essential role of Dol-P in protein glycosylation (Kornfeld and Kornfeld, 1985). Solely, Griffiths and co-workers have configured their MS setup to such an extent that it enabled the detection of Dol-sulphates and Dol-P from standard mixtures (Griffiths, et al., 1996). Furthermore, desorption electron impact and desorption chemical ionization MS spectra of mycobacterial decaprenyl-P have been recorded (Wolucka and de Hoffmann, 1994).

The recent discovery of inherited human disorders of Dol-P and Dol-P linked oligosaccharide biosynthesis (Ohkura, et al., 1997; Imbach, et al., 2000b; Kranz, et al., 2007b) calls for an accurate and quantitative method to measure variations of Dol-P levels in minute amounts. Here, we present the first detailed analysis of Dol-P at the cell culture scale by electrospray ionization MS (ESI-MS), which enabled the determination of Dol-P composition as well as the saturation state of the α-isoprene units. In addition, a HPLC method was adapted to allow the quantitative analysis of Dol-P isolated from cell cultures.

Materials and Methods

Materials - C_{80}-polyprenol-P and C_{95}-Dol-P standards were purchased from Larodan Fine Chemicals (Sweden). The mixed Dol-P standard and the fluorophore 9-anthryldiazomethane (9-ADM) were from Sigma-Aldrich (Switzerland). Acetonitrile (Scharlau, Spain), dichloromethane (Sigma-Aldrich) and *n*-hexane (Scharlau) were at least of HPLC grade. Residual reagents were of analytical grade.

Cell culture - HeLa cells were cultured in DMEM (Invitrogen, USA) supplemented with 10% fetal calf serum (Bioconcept, Switzerland) under 5% CO_2 at 37°C. During statin treatment, the cells were grown for 72 hours in medium containing 50 µM pravastatin (Calbiochem, USA) and the medium was changed every 24 hours.

Extraction, hydrolysis and purification of Dol and Dol-P - The extraction protocol of Elmberger and coworkers set up for tissue samples (Elmberger, et al., 1989) was adapted for cultured cells. Approximately 3 x 10^8 HeLa cells were released by trypsinization, washed once with medium and resuspended in 6 ml H_2O. Prior to the addition of 6 ml MeOH to the sample, 25 µg of C_{80}-polyprenol-P were added as internal standard. Alkaline hydrolysis and subsequent partitioning were performed as described (Elmberger, et al., 1989). Briefly, the cell suspension was hydrolyzed by addition of 3 ml 15 M KOH and heated at 100°C for 60 min. After partitioning by addition of 6 ml MeOH and 24 ml dichloromethane, residual Dol-ester were further hydrolyzed at 40°C for 60 min. The lower phase was washed five times with Folch upper phase (dicholomethane / MeOH / H_2O; 3:48:47) and evaporated to dryness.

Purification and separation of Dol and Dol-P were accomplished according to Elmberger *et al.* (Elmberger, et al., 1989). Briefly, Dol and Dol-P were purified on a C_{18} Sep Pak column (Waters)

followed by their separation on a Silica Sep Pak column (Waters).

Dimethylation and selective demethylation of Dol-P - Dol-P were dissolved in 3 ml diethylether and given to the outside tube of an Aldrich diazomethane generator system (Sigma-Aldrich). In the inside tube, 0.35 g diazald (Sigma-Aldrich) were suspended in 1 ml carbitol (Sigma-Aldrich). After assembly of the two parts and cooling in an ice bath, 1.5 ml 37% KOH were added dropwise to the inner tube. The resulting excess of diazomethane could be observed as yellow staining of the diethylether phase. After an incubation of 60 min on ice, the inside tube was removed and dimethylation of Dol-P was continued in the outside tube at room temperature for 2 h. The reaction was stopped by evaporation of the diethylether under a stream of nitrogen. The reaction products were dissolved in 4 ml of *tert*-butylamine (Sigma-Aldrich) and incubated for 14 h at 70°C to attain selective demethylation of dimethylated Dol-P (Yamada, et al., 1986). After evaporation of the *tert*-butylamine, monomethylated Dol-P were acidified by addition of 1.5 ml of 0.1 *N* HCl, subsequently extracted three times with 3 ml of diethylether and evaporated to complete dryness.

9-ADM labelling - Monomethylated or untreated Dol-P were labelled with the fluorophore 9-ADM according to the protocol of Yamada and coworkers (Yamada, et al., 1986). Briefly, the sample was dissolved in 600 µl of diethylether saturated with 9-ADM and incubated for 6 h on ice in the dark. The reaction was stopped by evaporation of the diethylether, and unreacted 9-ADM was separated from the reaction products by organic extraction (Yamada, et al., 1986). Labelled Dol-P were dissolved in 100 µl of acetonitrile/dichloromethane (3:2).

HPLC - Chromatographic separation was performed using a LaChrom D-7000 HPLC system (Merck, Germany) equipped with an Inertsil ODS-3 column (5 µm, 4.6 x 250 mm; GL Sciences Inc., Japan) including a pre-column and a LaChrom L-7485 fluorescence detector. Isocratic elution in acetonitrile / dichloromethane (3:2) containing 0.01% diethylamine (Sigma-Aldrich) was carried out at a flow rate of 1 ml/min at 30°C (Yamada, et al., 1986). Fluorescence was detected by excitation at 365 nm and emission at 412 nm.

Mass spectrometry - Negative ion mass spectrometry was performed on a 3200 QTRAP mass spectrometer equipped with a nanoelectrospray ion source (NanoSpray II; Applied Biosystems, USA) and an Eksigent NanoLC-2D system (Eksigent Technologies, USA). Samples were dissolved in dissolution solvent (90% acetonitrile, 10% *n*-hexane, 0.01% diethylamine) and directly injected into a 10 µl injection loop. After loading, the loop was switched in line a 20 µm ID, fused silica capillary, connected to the emitter tip of the nanoelectrospray ion source. The sample was introduced to the ion source using the dissolution solvent at a flow rate of 0.4 µl / min. All mass spectra were acquired manually in ion trapping modes and for the Enhanced MS scans the following settings were applied: Curtain Gas flow: 10 psi, collision gas pressure: high, ion spray voltage: between -2400 V and -4500 V, interface heater temperature: 150°C, declustering potential: -200 V, entrance potential: -10 V, collision energy -10 V. Negative ion fragment spectra were acquired in Enhanced Product Ion mode and the collision energy was set to -100 V.

Results

The detection of Dol-P by direct infusion negative ion nano electronspray MS was first demonstrated of C_{95}-Dol-P standards ($C_{95}H_{156}PO_4^-$; ω-t_2-c_{15}-S-P). C_{95}-Dol-P could be monitored at m/z 1392.5 (Fig. 1A), which is consistent with the theoretical mass of 1392.17 Da. The commercially available standards also contained small amounts of C_{90}-Dol-P ($C_{90}H_{148}PO_4^-$; 1324.11 Da) and C_{100}-Dol-P ($C_{100}H_{164}PO_4^-$; 1460.24 Da), which could be observed at m/z 1324.4 and 1460.6, respectively (Fig. 1A). To confirm the identity of the C_{95}-Dol-P, the species at m/z 1392.5 was subjected to collision induced fragmentation. The resulting MS/MS spectrum (Fig. 1B) showed a major fragment series starting at m/z 164.9 (Table 1). The 164.9 Da fragment ion was assigned to reduced monoprenyl-P $C_5H_{10}PO_4^-$ (Fig. 1B insert) featuring a mass of 165.03 Da. Additional minor fragment series were also observed in the MS/MS spectrum with the starting ions at m/z 122.8 and 178.9, respectively (Fig. 1B). All three decay series depicted the sequential elimination of single isoprene (C_5H_8; 68.06 Da) units (D'Alexandri, et al., 2006) (Table 1), thereby validating the assignments of the polyprenol C_{95}-Dol-P standard.

The saturated C_{80}-polyprenol-P standard ($C_{80}H_{130}PO_4^-$; ω-t_2-c_{13}-P) was also analyzed by ESI-MS. The resulting spectrum showed a main peak at m/z 1186.7, which corresponded to hexadeca-prenyl-P with the theoretical mass of 1185.97 Da (Fig. 1C). The signals at m/z 1202.6 and 1218.4 differed from the main signal by 16 Da increments, which corresponded to oxidized forms of the C_{80}-polyprenol-P. The spectrum showed that the standard was not completely monomolecular, since traces of C_{75}-polyprenol-P ($C_{75}H_{122}PO_4$-; 1117.91 Da) were recorded at m/z 1120.2 (Fig. 1C). Fragmentation of the C_{80}-poly-prenol-P yielded a similar decay series (Fig. 1D) as observed for the C_{95}-Dol-P (Fig. 1B), thereby con-

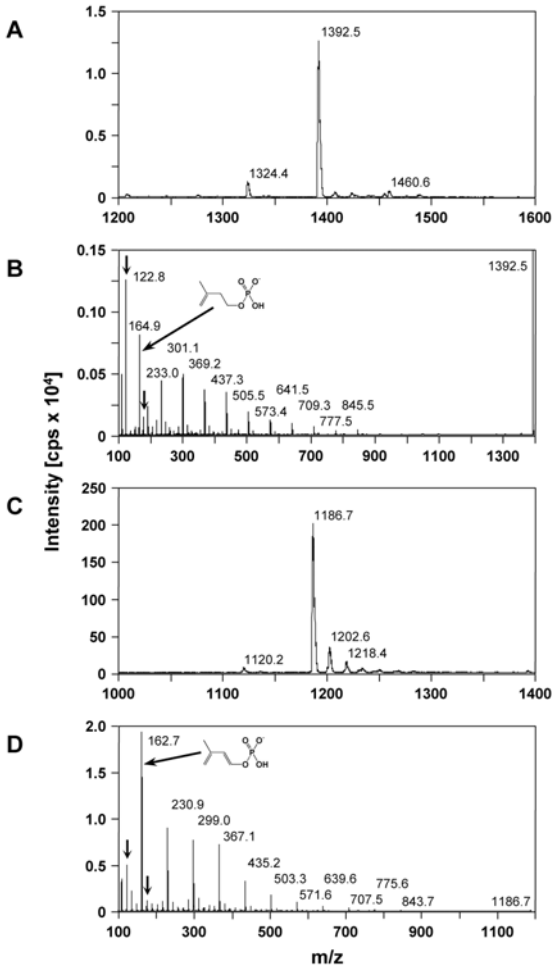

Figure 1: MS detection of C_{95}-Dol-P and C_{80}-polyprenol-P standards. **(A)** The negative ion MS spectrum of C_{95}-Dol-P shows the expected species with a mass of 1392.17 Da together with small amounts of co-purified C_{90}- and C_{100}-Dol-P. **(B)** The C_{95}-Dol-P at m/z 1392.5 was subjected to collision induced fragmentation and a major fragment ion series started at m/z 164.9 with the $C_5H_{10}PO_4^-$ ion (structure inserted). Further fragments incrementing by 68 Da were detected, consistent with the elimination of single isoprene units from the C_{95}-Dol-P precursor (Table 1). Starting ions of minor fragment series are indicated with an arrow. **(C)** The C_{80}-polyprenol-P standard yielded the expected ion at m/z 1186.7, whereas traces of C_{75}-polyprenol-P and oxidized forms of the hexadecaprenyl-P standard were also detected in the MS spectrum. **(D)** Decay sequences were recorded after the collision induced fragmentation of the main C_{80}-polyprenol-P ion at 1186.7. The primary ion $C_5H_8PO_4^-$ (structure inserted) of the major fragmentation series represents unsaturated mono-

prenyl-P. The eliminated fragments are identical to those observed for C_{95}-Dol-P in **(B)** (Table 2). The starting ion of minor decay series are indicated with arrows.

firming its polymeric isoprene composition. As expected, the entire fragment ion series starting at m/z 162.7 ($C_5H_8PO_4^-$, 163.02 Da calculated, Fig. 1D) was shifted by 2 Da due to the saturated α-isoprene unit of the C_{80}-polyprenol-P (Table 2).

Table 1. Major decay ion series resulting from the fragmentation of C_{95}-Dol-P.

Isoprene units	Molecular formula	Theoretical mass [Da]	Detected mass [m/z]
1	$C_5H_{10}PO_4^-$	165.03	164.9
2	$C_{10}H_{18}PO_4^-$	233.09	233.0
3	$C_{15}H_{26}PO_4^-$	301.16	301.1
4	$C_{20}H_{34}PO_4^-$	369.22	369.2
5	$C_{25}H_{42}PO_4^-$	437.28	437.3
6	$C_{30}H_{50}PO_4^-$	505.34	505.5
7	$C_{35}H_{58}PO_4^-$	573.41	573.4
8	$C_{40}H_{66}PO_4^-$	641.47	641.5
9	$C_{45}H_{74}PO_4^-$	709.53	709.3
10	$C_{50}H_{82}PO_4^-$	777.60	777.5
11	$C_{55}H_{90}PO_4^-$	845.66	845.5
19	$C_{95}H_{156}PO_4^-$	1392.17	1392.5

In a second approach, the C_{95}-Dol-P standards was labelled with 9-ADM without prior treatment. The purified products (Fig. 2C, insert) were separated isocratically by HPLC. The resulting fluorescent profile showed the elution of 9-ADM labelled C_{95}-Dol-P as a sharp peak at a retention time of 65.1 min (Fig. 2A). Notably, two additional fluorescent species were observed eluting at 52.8 min and 80.7 min. Considering the ESI-MS spectrum of unlabelled C_{95}-Dol-P standards (Fig. 1A), these peaks likely represented 9-ADM labelled C_{90}- and C_{100}-Dol-P. The ESI-MS analysis of the collected main peak confirmed the expected composition of the product with an m/z of 1582.5 ($C_{110}H_{166}PO_4^-$; 1582.25 Da calculated), while only traces of unlabelled standards could be detected at m/z 1392.7 (Fig. 2C). The C_{80}-polyprenol-P standard was used as spiking compound to quantify the levels of Dol-P in cell culture samples. The analysis of 2 µg of 9-ADM labelled C_{80}-polyprenol-P (Fig. 2D, insert) by HPLC yielded a peak eluting at a retention time of 31.6 min (Fig. 2B). The identity of this peak was confirmed by ESI-MS recording (Fig. 2D), which produced the expected ion at m/z 1376.3 ($C_{95}H_{140}PO_4^-$; 1376.05 Da).

Table 2. Major decay ion series resulting from the fragmentation of C_{80}-polyprenol-P.

Isoprene units	Molecular formula	Theoretical mass [Da]	Detected mass [m/z]
1	$C_5H_8PO_4^-$	163.02	162.7
2	$C_{10}H_{16}PO_4^-$	231.08	230.9
3	$C_{15}H_{24}PO_4^-$	299.14	299.0
4	$C_{20}H_{32}PO_4^-$	367.20	367.1
5	$C_{25}H_{40}PO_4^-$	435.27	435.2
6	$C_{30}H_{48}PO_4^-$	503.33	503.3
7	$C_{35}H_{56}PO_4^-$	571.39	571.6
8	$C_{40}H_{64}PO_4^-$	639.45	639.6
9	$C_{45}H_{72}PO_4^-$	707.52	707.5
10	$C_{50}H_{80}PO_4^-$	775.58	775.6
11	$C_{55}H_{88}PO_4^-$	843.64	843.7
16	$C_{80}H_{130}PO_4^-$	1185.97	1186.7

To validate the ability of the methods to resolve Dol-P of varying lengths, a mixed Dol-P standard comprising C_{85} - C_{105}-Dol-P, which are typical for

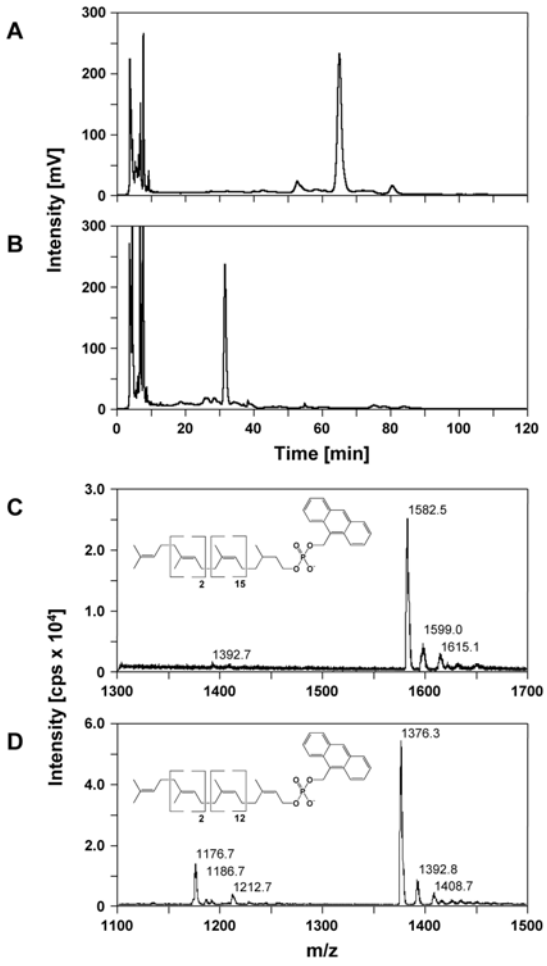

Figure 2: HPLC and MS analysis of 9-ADM labelled C_{95}-Dol-P and C_{80}-polyprenol-P standards. **(A)** The 9-ADM labelled C_{95}-Dol-P standard produced a profile with a main peak eluting at 65.1 min and smaller peaks of C_{90}- and C_{100}-Dol-P at 52.8 and 80.7 min. **(B)** The 9-ADM labelled C_{80}-polyprenol-P standard eluted at a retention time of 31.6 min in the HPLC profile. **(C)** The 9-ADM labelled C_{95}-Dol-P peak (structure inserted) was collected from the HPLC run and subjected to MS analysis, yielding a principal ion at m/z 1582.5 and minor ions at m/z 1599.0 and 1615.1, representing oxidation products with the typical 16 Da mass increase. **(D)** The formation of 9-ADM labelled C_{80}-polyprenol-P (structure inserted) was confirmed by MS by detecting a main product at the expected mass at m/z 1376.3.

mammalian cells (Chojnacki and Dallner, 1988), was labelled with 9-ADM and separated by HPLC. A dimethylation and subsequent selective demethylation was included in the Dol-P preparation to improve product stability as established by Yamada *et al.* (Yamada, et al., 1986). The fluorescent profile of monomethylated and 9-ADM labelled mixed Dol-P standard showed a clean separation of C_{85}-Dol-P, C_{90}-Dol-P, C_{95}-Dol-P, C_{100}-Dol-P and C_{105}-Dol-P (Fig. 3A). The ESI-MS analysis of the unlabelled mixed Dol-P standard confirmed the presence of Dol-P species in comparable ratios to those detected by HPLC (Fig. 3C, i.e. C_{85}-Dol-P at m/z 1257.2, C_{90}-Dol-P at m/z 1325.1, C_{95}-Dol-P at m/z 1393.1, C_{100}-Dol-P at m/z 1461.0, and C_{105}-Dol-P at m/z 1528.9).

To investigate the Dol-P pool of cultured cells, we prepared batches of 2.0 x 10^8 HeLa cells. After purification and separation of Dol-P from polyprenol alcohols, 75% of the samples were labelled with 9-ADM and analyzed by HPLC. The resulting chromatograms showed that Dol-P in HeLa cells mainly consisted of C_{95}-Dol-P (Fig. 3B), which was in agreement with earlier reports on various human tissues (Tollbom and Dallner, 1986; Andersson, et al., 1987). Additionally, C_{90}- and C_{100}-Dol-P were detected, but to a lower extend (Fig. 3B). By contrast, the C_{85}- and the C_{105}-Dol-P species were either completely absent or their amounts were below the detection level.

The remaining 25% of the purified Dol-P pool from HeLa cells were subjected to nano-ESI-MS analysis without further treatment. Again, the main species C_{95}-Dol-P at m/z 1392.7 was flanked by lower amounts of C_{90}-Dol-P at m/z 1324.8 and C_{100}-Dol-P at m/z 1460.8 (Fig. 3D). The enhanced sensitivity of nano-ESI-MS compared to HPLC allowed the detection of minimal amounts of C_{85}- and C_{105}-Dol-P at m/z 1256.3 and 1529.0, respectively. To allow the quantification of Dol-P levels in HeLa cells, 25 µg of C_{80}-polyprenol-P standard were added to each cell samples before extraction. Dol-P and polyprenol-P were separated

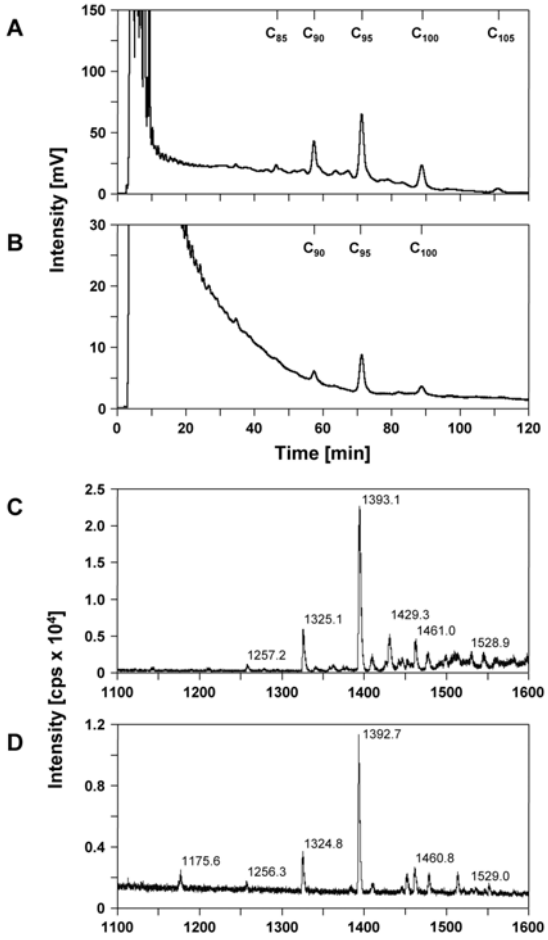

Figure 3: HPLC and MS analysis of mixed Dol-P standard and Dol-P in HeLa cells. **(A)** A mixed Dol-P standard was monomethylated, labelled with 9-ADM and subsequently separated by HPLC. The distinct Dol-P peaks were identified and marked at the top of the chromatograms. **(B)** The Dol-P pool of HeLa cells was analyzed by HPLC and yielded the main compound C_{95}-Dol-P and smaller amounts of C_{90}- and C_{100}-Dol-P. **(C)** The MS spectrum of unlabelled mixed Dol-P standard confirms the presence of C_{85}-, C_{90}-, C_{95}-, C_{100}- and C105-Dol-P at m/z 1257.2, 1325.1, 1393.1, 1461.0 and 1528.9, respectively. **(D)** The HeLa Dol-P pool yielded an MS profile demonstrating the presence of C_{85}-, C_{90}-, C_{95}-, C_{100}- and C_{105}-Dol-P at m/z 1256.3, 1324.8, 1392.7, 1460.8 and 1529.0, respectively.

by HPLC and the corresponding peak areas compared (Fig. 4A). An amount of 1.43 µg Dol-P per 10^8 HeLa cells was calculated, which represented the sum of 0.21 µg of C_{90}-Dol-P, 0.81 µg of C_{95}-Dol-P, 0.40 µg of C_{100}-Dol-P and 0.01 µg of C_{105}-Dol-P (Fig 4C). The linearity of Dol-P quantification was confirmed by measuring increasing ratios of spiked C_{80}-polyprenol-P standard to HeLa cell extracts (Fig. 4D).

Dol and cholesterol share the same biosynthetic pathway until the formation of the C_{15}-farnesyl-pyrophosphate intermediate (Swiezewska and Danikiewicz, 2005). Hence, statins inhibiting the 3- hydroxy-3- methyl- glutaryl- CoA reductase enzyme do not only act as cholesterol lowering drugs, but also affect the synthesis of Dol and Dol-P (Astrand, et al., 1986; Appelkvist, et al., 1993). To investigate the impact of statins on the level and composition of the Dol-P pool of HeLa cells, we added 50 µM pravastatin (Koga, et al., 1990; McTavish and Sorkin, 1991) to the cell culture medium for 72 h. The separation of the resulting 9-ADM labelled Dol-P by HPLC showed an overall reduction of Dol-P, which was more pronounced for Dol-P species of large isoprene chain lengths (Fig. 4B). The pravastatin treatment decreased C_{90}-Dol-P to 0.20 µg per 10^8 HeLa cells, whereas C_{95}-Dol-P was reduced to 0.29 µg per 10^8 cells. Only minor amounts of C_{100}-Dol-P, below 0.01 µg per 10^8 cells were detected (Fig. 4C). The overall levels of Dol-P were reduced to 0.50 µg per 10^8 cells, representing 35% of the normal Dol-P levels in HeLa cells.

Discussion

The present study describes two methodological approaches to analyse and quantify low amounts of Dol-P from cultured human cells. Recording of negative ion ESI-MS spectra allowed us to determine the isoprene chain length and the saturation state of the α-isoprene of mixed polyprenol-P samples. In addition, the separation of 9-ADM labelled Dol-P and polyprenol-P by HPLC enabled the quantitative determination of

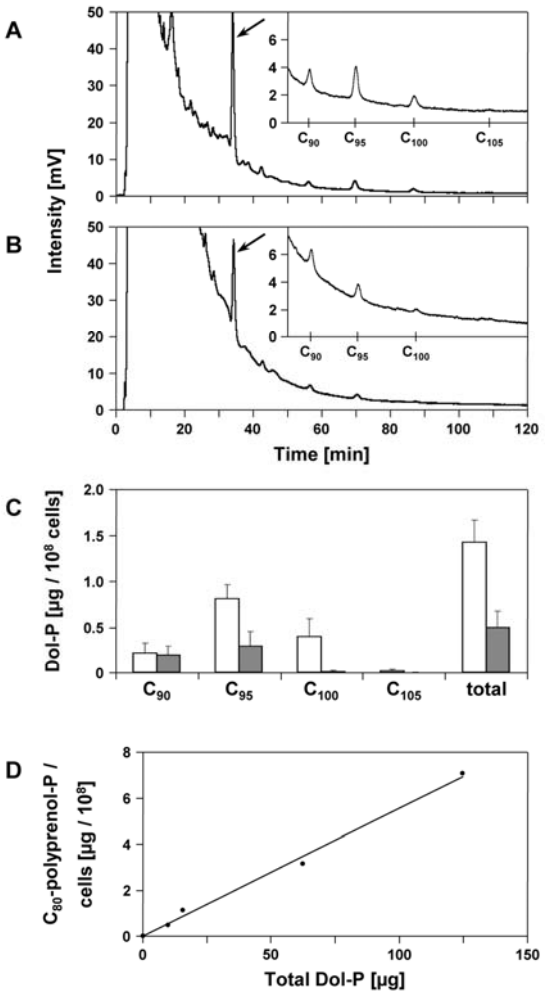

Figure 4: Quantification of Dol-P from mock- and pravastatin-treated HeLa cells. **(A)** Dol-P from mock-treated HeLa cells were prepared in presence of 25 µg C_{80}-polyprenol-P as internal standard. Individual Dol-P were assigned in the enlarged section of the profile and quantified by comparison with hexadecaprenyl-P eluting at 34.0 min (marked by arrow). **(B)** HPLC profile of pravastatin-treated HeLa cells including a C_{80}-polyprenol internal standard (marked by arrow). **(C)** The Dol-P levels from four independent experiments were quantified and normalized to 10^8 HeLa cells. White bars represent Dol-P levels from mock-treated cells and gray bars Dol-P levels from pravastatin treated cells. Error bars show standard derivations. **(D)** Linear correlation of C_{80}-polyprenol-P standard to HeLa cell extract of four independent experiments against measured Dol-P amounts, linear regression coefficient $R^2 = 0.9943$.

Dol-P by comparison with known amounts of C_{80}-polyprenol-P internal standard.

The limited sample volume of cultured human cell renders the detection of Dol-P a challenging task. To ensure a reliable measurement, the application of sensitive methods such as ESI-MS and fluorescence detection is indispensable. Along this line, the direct UV-detection of Dol-P established by Elmberger *et al.* (Elmberger, et al., 1989) is not applicable at the cell culture scale. Earlier approaches also applied metabolic labelling of the polyprenol chain with [^{3}H]-acetic acid or -mevalonate (Ekstrom, et al., 1984; Keller, et al., 1989) or fluorescent labelling of the phosphate group (Yamada, et al., 1986; Jiang, et al., 1993). The disadvantages of isotopic labelling, such as interference of exogenously added intermediates on Dol biosynthesis or variable incorporation of the isotopes, significantly impair the quantitative determination of Dol-P. The fluorescent labelling of phosphate groups enables both the sensitive detection and the reliable quantitative measurement of Dol-P in cell cultures. In this context, the fluorophore 9-ADM has the advantage of being commercially available in contrast to other compounds applied earlier (Yamada, et al., 1986). However, the drawback of 9-ADM labelling itself was a poor reproducibility because of high susceptibilty to hydrolysis. To increase the stability of the compounds during the labelling procedure, Dol-P samples were first dimethylated and selectively demethylated (Yamada, et al., 1986). This step dramatically improved the stability and reproducibility of the procedure, which once optimized enabled the detection of Dol-P at the ng range (Fig. 4).

In all ESI-MS spectra recorded, additional signals incrementing with 16 Da could be observed. We assume that these signals were generated by oxidation of double bonds within the isoprenes,

either by spontaneous epoxidation (Gnanadesikan and Corey, 2008) or by [2 + 2] cycloaddition of molecular oxygen (Griesbeck, et al., 2003). This phenomenon has already been observed earlier (Griffiths, et al., 1996) and we found it more pronounced when derivatizing polyprenol-P. Constant sample storage in darkness, at -20°C and under inert atmosphere helped at keeping the oxidation to low levels.

The present combined approach can be used for the analysis of Dol-P in cells under various pathological conditions. Recently, the first inherited human disorder affecting the Dol-kinase and hence Dol-P biosynthesis has been described (Kranz, et al., 2007b). Since disrupted Dol phosphorylation has severe implications on protein N-glycosylation, Dol-kinase deficiency is counted as a form Congenital Disorders of Glycosylation (CDG). It is likely that additional genes from the Dol-P biosynthetic pathway, such as the *cis*-prenyltransferase, the polyprenol mono- or pyrophosphatases or the polyprenol-reductase (Ohkura, et al., 1997) may soon be found as cause of CDG. Accordingly, the present technique represents an ideal screening tool for the detection of truncated or incompletely saturated Dol-P in patient derived fibroblasts.

We did test our Dol-P quantification method by evaluating the effect of the 3-hydroxy-3-methyl-glutaryl-CoA reductase inhibitor pravastatin (Koga, et al., 1990) on Dol-P levels in HeLa cells. The statin leads to a striking decrease of Dol-P levels in addition to its action on cholesterol biosynthesis. The overall reduction to 35% of control levels mostly results from by the loss of large Dol-P species. The reason why large Dol-P are more susceptible to pravastatin treatment is unclear but it ismlikey that *cis*-prenyltransferase leads to shorter polyprenols species under conditions of limited mevalonate availability. Along this line, part of the numerous side effects of pravastatin (McClure, et al., 2007) could be explained by inhibition of the diverging isoprenoid pathways leading to the formation of Dol, ubiquinone or prenylated proteins (Swiezewska and Danikiewicz, 2005). Low levels of free Dol and Dol-P might lead to eczema, itching and loss of hair, as observed in Dol-kinase deficient patients (Kranz, et al., 2007b). Reduced N-linked and O-mannose glycosylations due to limited amounts of the membrane anchor Dol-P might contribute to muscle and liver problems.

In conclusion, the introduced methodology provides a reliable analytical setup to address the impact of drugs and other treatments on Dol-P in cell culture models and to identify novel inherited disorders affecting Dol-P biosynthesis.

Acknowledgements

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5 Zaragozic acid A improves the molecular indications of Congenital Disorders of Glycosylation in patient fibroblasts

(Manuscript designed for submission to the Journal of Biological Chemistry)

Contribution:

Toxicity determination
Dolichol-phosphate analysis
Dolichol-phosphate-mannose analysis
CD59 flow cytometry
LLO analysis
NLO analysis

Zaragozic acid A improves the molecular indications of Congenital Disorders of Glycosylation (CDG) in patient fibroblasts

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Running head: Zaragozic acid A improves N-glycosylation

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Congenital Disorders of Glycosylation (CDG) constitute a group of inherited human diseases entailed by deficient protein glycosylation. Mutations within genes that encode enzymes involved in the biosynthesis of the dolichol-linked N-glycosylation precursor $GlcNAc_2Man_9Glc_3$ constitute the major group of CDG. Most of the mutations leave residual catalytic activity to the affected enzymes. In theory, the efficiency of such K_M mutations should be increased by elevated substrate disposability. Hence, allocation of dedicated donor substrates could improve deficient N-glycosylation observed in CDG. The squalene synthase inhibitor zaragozic acid A (ZA) was found to induce the biosynthesis of dolichol-phosphate (Dol-P) besides acting as cholesterol lowering agent. Dol-P serves as membrane anchor for both, monosaccharide donors and the oligosaccharide precursor required for N-glycosylation. ZA induced stimulation of control and Dol-P-Man synthase subunit 1 deficient (DPM1-CDG) patient fibroblasts lead to increased formation of Dol-P-Man. Furthermore, disease related accumulations of Dol-linked oligosaccharide intermediates could be diminished upon ZA administration and simultaneously, wild-type levels of complete oligosaccharide precursor could be accomplished. Apart from under-occupancy of N-glycosylation sites in target glycoproteins, the transfer of fragmentary oligosaccharides assigns a characteristic of most CDG. ZA treatment considerably improves the ratio of pathologically protein-linked glycans to naturally N-glycosylated proteins. Since Dol-P-Man serves likewise as donor substrate for O-mannosylation and glycosylphosphatidylinositol (GPI) - anchor formation, DPM1-CDG patients suffer from reduced cell surface expression of GPI-anchored proteins. However, ZA administration restores wild-type expression levels of a model GPI-anchored protein. Consequently, ZA has the ability to correct the molecular indications of CDG appearing in patient fibroblasts.

Footnotes

[a] Current address: Amgen Switzerland AG, Zählerweg 6, CH-6301 Zug, Switzerland

[b] The abbreviations used are: CDG, Congenital Disorders of Glycosylation; Dol, dolichol; GPI, glycosylphosphatidylinositol; HMG-CoA, 3-hydroxy-3-methyl-glutaryl Coenzyme A; LLO, lipid-linked oligosaccharides; NLO, N-linked oligosaccharides; ZA, zaragozic acid A

Introduction

Inherited dysfunctions of protein glycosylation cause a series of human diseases termed Congenital Disorders of Glycosylation (CDG)[b] (Freeze, 2006). Patients suffer from a broad range of severe multi-systemic symptoms, which can be divided into developmental defects, marked by impaired central nervous system development and dysmorphic features, and physiological disorders affecting for instance coagulation or hormonal balances (Leroy, 2006). Mutations in genes coding either for enzymes that are involved in the biosynthesis of the lipid-linked oligosaccharide (LLO) required for N-glycosylation (Jaeken and Matthijs, 2007) or for members of the glycan processing (De Praeter, et al., 2000; Hansske, et al., 2002) or transport machinery of N-glycoproteins (Foulquier, 2008) form the molecular basis of CDG. Disorders affecting the assembly of the LLO precursor dolichol-pyrophosphate (Dol-PP)-$GlcNAc_2Man_9Glc_3$ are comprised in the major group of

biosynthetic defects and lead to under-occupancy of N-glycosylation sites in target glycoproteins (Haeuptle and Hennet, 2009).

The successive biosynthesis of the oligosaccharide precursor proceeds first on the cytoplasmatic and after translocation on the luminal side of the endoplasmic reticulum (Kornfeld and Kornfeld, 1985), whereat the polyisoprene Dol-P serves as membrane anchor (Schenk, et al., 2001a). Thus, Dol-P serves not only as carrier of maturing LLO, but also as lipid component of Dol-P-Man and Dol-P-Glc, both donor substrates for luminally acting mannosyl- and glucosyltransferases. Notably, a deficiency of the Dol-P-Man synthase subunit 1 (DPM1-CDG) leads to disturbed protein glycosylation and glycosylphosphatidylinositol (GPI)-anchor formation (Imbach, et al., 2000b), since both posttranslational modifications rely on Dol-P-Man (Maeda and Kinoshita, 2008).

Recently, the group of CDG was expanded by the first defect affecting the biosynthesis of the membrane anchor Dol-P (Kranz, et al., 2007b). The lethal outcome of four Dol-kinase deficient patients confirmed the fundamental role of the polyprenyl-P in human protein N-glycosylation. Its biosynthesis follows the sterol pathway until the accomplishment of the C_{15}-intermediate farnesyl-PP (Swiezewska and Danikiewicz, 2005) (Fig. 1A). Instead of squalene formation by head to head assembly of two farnesyl-PP molecules (Muscio, et al., 1974), the consecutive condensation of isopentenyl-PP units leads to the diverging synthesis of polyprenyl-PP, a pre-stage of Dol-P. Hence, the squalene synthase catalyses the first reaction that yields exclusively to the formation of sterol compounds, such as cholesterol and steroid hormones (Fig. 1A).

Inhibition of squalene synthase by the statin zaragozic acid A (ZA) leads to the stimulation of prior diverging pathways, and hence to increased

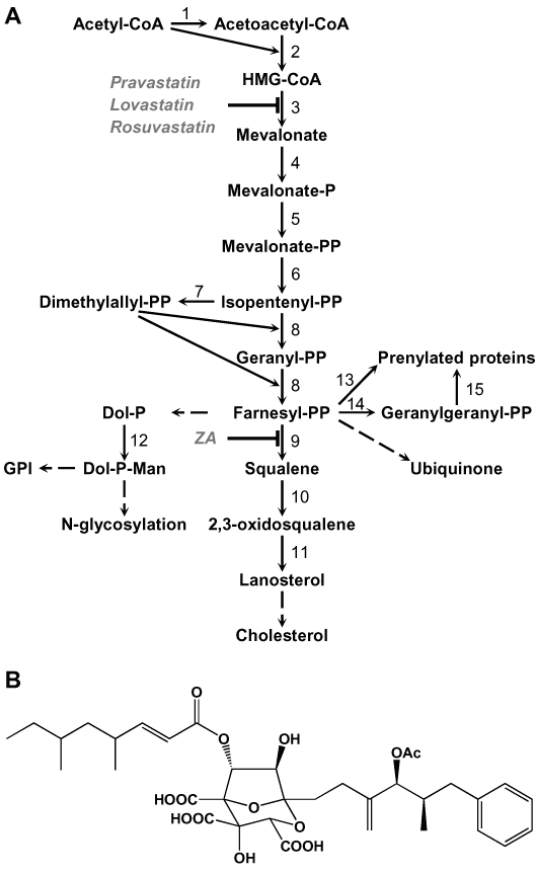

Figure 1: Statins acting on the sterol biosynthesis pathway. **(A)** The biosynthetic formation of sterols, such as cholesterol, starts from acetyl-CoA and leads via the C_{15}-intermediate farnesyl-PP to its various endproducts. The involved enzymes are numbered like follows: (1) acetoacetyl-CoA thiolase, (2) HMG-CoA synthase, (3) HMG-CoA reductase, (4) mevalonate kinase, (5) phosphomevalonate kinase, (6) mevalonate-PP decarboxylase, (7) isopentenyl-PP isomerase, (8) farnesyl-PP synthase, (9) squalene synthase, (10) squalene monooxygenase, (11) squalene epoxydase, (12) Dol-P-Man synthase, (13) protein farnesyl-transferase, (14) geranylgeranyl-PP synthase, (15) protein geranylgeranyltransferase. At the point of farnesyl-PP, the pathway diverges to the formation of prenylated proteins, ubiquinone or Dol. The poly-isoprene Dol-P is incorporated into Dol-P-Man, a key intermediate in protein glycosylation and GPI-anchor formation. While pravastatin, lovastatin and rosuva-statin lower cellular cholesterol levels by inhibiting the pathway at the stage of the HMG-CoA reductase, ZA achieves this by inhibiting the squalene synthase. The chemical structure of ZA is depicted in **(B)**.

formation of Dol and Dol-P (Keller, 1996). ZA, also known as squalestatin I, was primary discovered by screening metabolites of filamentous fungi for cholesterol lowering activity (Bergstrom, et al., 1995). Detailed analysis disclosed that ZA acts as competent inhibitor of the squalene synthase by mimicking the farnesly-PP substrate or the stable intermediate presqualene-PP with its bicyclic, highly acidic core (Fig. 1B). In contrast to statins acting on mutual steps of both, Dol and cholesterol biosynthesis, such as the 3-hydroxy-3-methyl-glutaryl coenzyme A (HMG-CoA) reductase inhibitors pravastatin (Koga, et al., 1990), lovastatin (Alberts, et al., 1980) or rosuvastatin (McTaggart, et al., 2001), ZA does not negatively affect the diverging pathways generating Dol, ubiquinone or prenylated proteins (Fig. 1A).

The property of the squalene synthase inhibitor as Dol biosynthesis stimulant in rats (Keller, 1996) raises rather the question whether ZA treatment might likewise increase Dol-P and therewith positively influence the thereof dependent N-glycosylation in CDG fibroblasts. This is likely the case, since most of the CDG are caused by K_M mutations leaving residual catalytic activity to the mutated enzymes (Grunewald, et al., 2001; Westphal, et al., 2001c). Noteworthy, this potential therapeutical approach appears to be in particular requisite, regarding that so far only one biosynthetic CDG subtype can effectively be treated. Thereby, orally applied Man allows the functional bypass of the defective isomerisation reaction in Man phosphate isomerase (MPI-CDG) patients (Niehues, et al., 1998; Westphal, et al., 2001b).

Experimental procedures

Materials: ZA was a generous gift from Merck & Co., Inc. (USA). The C_{80}-polyprenyl-P standards were purchased from Larodan Fine Chemicals (Sweden), and broad range mammalian Dol-P standards were from Sigma-Aldrich (Switzerland). Acetonitrile (Scharlau, Spain), dichloromethane (Sigma-Aldrich) and water (Sigma-Aldrich) were of HPLC grade, other chemicals were of analytical grade.

Cell culture: Skin fibroblasts were cultured in DMEM (Invitrogen, USA) supplemented with 10% fetal calf serum (Bioconcept, Switzerland) under 5% CO_2 at 37°C. ZA was dissolved in DMSO (Sigma-Aldrich) at a concentration of 25 mM and administered to the fibroblasts for variable times and concentrations as indicated for the respective experiments, whereas ZA containing medium was exchanged every 24 h.

Dol-P analysis: Fibroblasts (approximately 4 x 10^7) were treated for 72 h with either 100 µM ZA or equivalent amounts of DMSO as control and harvested by trypsinization. The washed cell pellet was dissolved in 12 ml of water/methanol (1:1). Prior to alkaline hydrolysis (Elmberger, et al., 1989), 15 µg of C_{80}-polyprenyl-P standards were added. The extracted Dol and Dol-P were purified on a C_{18} Sep Pak column (Waters, USA) and subsequently separated on a Silica Sep Pak column (Waters) according to Elmberger and colleagues (Elmberger, et al., 1989). The Dol-P were dimethylated using diazomethane generated in an Aldrich diazomethane generator system (Sigma-Aldrich) according to the manufacturer's instructions. The resulting Dol-P-Me_2 were selectively demethylated by overnight incubation in *tert*-butylamine (Sigma-Aldrich) at 70°C (Yamada, et al., 1986). Monomethylated Dol-P samples were dissolved in a saturated solution of 9-anthryldiazomethane (Sigma-Aldrich) in diethylether and incubated for 6 h in darkness on ice. Anthracene labelled Dol-P were separated from non-reacted labelling agent by organic extraction (Yamada, et al., 1986). The purified

products were dissolved in acetonitrile/ dichloromethane (3:2) and subjected to HPLC on an Inertsil ODS-3 column (5 µm, 4.6 x 250 mm; GL Sciences Inc., Japan) equipped with a precolumn. Isocratic elution in acetonitrile/dichloromethane (3:2) and 0.01% diethylamine (Sigma-Aldrich) was performed at a flow rate of 1 ml/min (Yamada, et al., 1986). Fluorescent labelled Dol-P were detected by excitation at 365 nm and emission at 412 nm. Defined amounts of internal standard C_{80}-polyprenyl-P were used for quantification.

Determination of Dol-P-Man: Approximately 4 x 10^7 fibroblasts were grown for 72 h in DMEM containing low Glc (5 mM; Invitrogen), supplemented with 2% fetal calf serum and 100 µM ZA or the equivalent amount of DMSO as control. Low Glc medium was utilized to achieve improved Man incorporation (Körner, et al., 1998). Cellular Dol-P-Man was metabolically labelled, extracted and purified according to the protocol of Körner and co-workers (Körner, et al., 1998). Briefly, the fibroblasts were labelled by incubation in DMEM containing 0.5 mM Glc and 125 µCi [^{3}H]-Man (Hartmann Analytic, Germany) for 30 min. Dol-P-Man and LLO of small size were extracted once with chloroform/methanol (2:1) and the extraction was repeated twice with chloroform/methanol (3:2). The combined organic phases were dried and washed (Körner, et al., 1998). Thin-layer chromato-graphy on Silica gel 60 plates was performed in chloroform/ methanol/water (65:25:4). The plates were analyzed by radiography after signal enhancement with an EN^{3}HANCE spray (PerkinElmer, USA) and the areas containing Dol-P-Man were scraped and counted in a TRI–CARB 2900 TR liquid scintillation analyser (Packard, USA).

CD59 flow cytometry: Fibroblasts (2 x 10^5) were grown for 24, 48, 72 or 144 h in the presence of 0, 10, 50 or 100 µM ZA. After trypsinization, the cells were washed once in PBS containing 2% fetal calf serum and then incubated with a FITC conjugated mouse α-human CD59 antibody (BD Pharmingen, USA) diluted 1:100 in PBS containing 2% fetal calf serum for 20 min on ice (Imbach, et al., 2000b). Fibroblasts were washed and analyzed on a BD FACSCanto II flow cytometer (BD Biosciences, USA) equipped with BD FACSDiva software (BD Biosciences).

LLO analysis: The LLO of fibroblasts (approximately 1.5 x 10^7) treated for 72 h with either 100 µM ZA or the equivalent amount of DMSO as control were analysed as described (Haeuptle, et al., 2008). The cells were starved for 45 min in fetal calf serum and Glc free DMEM (Invitrogen) and metabolically labelled for 60 min by addition of 150 µCi [^{3}H]-Man. The LLO were extracted from cell pellets and the glycan moieties were released by mild acid hydrolysis in 0.1 *N* HCl. The oligosaccharides were purified by ion-exchange chromatography on AG1-X2 and AG50W-X8 resins (Bio-Rad, USA) and by hydrophobic chromatography on Supelclean ENVI-Carb 120/400 beads (Supelco, USA) and C_{18} Sep Pak columns and subjected to HPLC analysis.

NLO analysis: Fibroblasts (approximately 1.5 x 10^7) were treated for 72 h with 100 µM ZA or equivalent amounts of DMSO as control. The cells were starved for 90 min in MEM (Sigma-Aldrich) supplemented with 5% dialyzed fetal calf serum (Invitrogen) and subsequently metabolically labelled for 60 min with 100 µCi [^{3}H]-Man. The glycoproteins were denatured and the N-linked oligosaccharides were released by overnight incubation with N-glycosidase F (New England BioLabs, USA) (Grubenmann, et al., 2002). The released glycans were purified and analyzed by HPLC as described for the LLO. However, the anion exchange resin AG1-X2 was not applied during

NLO purification to avoid the loss of negatively charged oligosaccharides.

Results

To address the effect of ZA on N-glycosylation in CDG fibroblasts, the toxicity of the statin compound on the cell culture system had to be determined first of all. From the broadness of 15 biosynthetic CDG (Haeuptle and Hennet, 2009), fibroblasts deriving from a Dol-P-Man synthase subunit 1 deficient (DPM1-CDG) patient (Imbach, et al., 2000b) were selected as appropriate model, since they feature reduced amounts of donor substrate Dol-P-Man, pathogenic alterations of lipid-, as well as protein N-linked oligosaccharides and actually they depicted impaired cell surface expression of GPI anchored proteins (Imbach, et al., 2000b; Kim, et al., 2000). Hence, fibroblasts of a DPM1-CDG patient and age matching controls were cultivated for ten days in the presence of distinct concentrations of ZA and analysed every 24 h for drug induced cell deformations or growth retardations. In conclusion, ZA was found to be tolerated by fibroblasts up to a concentration of 125 µM (data not shown).

The effect of ZA on glycosylation was determined by measuring cellular levels of Dol-P, Dol-P-Man, a GPI-anchored model protein, LLO and NLO, all parameters affected by CDG. According to this, Dol-P of control fibroblasts were extracted, hydrolysed, purified, fluorescently labelled and finally separated by HPLC. The resulting fluorescent profile (Fig. 2A) was compared to the profile of Dol-P isolated from control fibroblasts treated for 72 h with 100 µM ZA (Fig. 2B). The pattern of Dol-P species changed upon ZA treatment, where particularly the formation of the longer C_{100}- and C_{105}-Dol-P appeared to be stimulated in ZA treated fibroblasts. Quantification of four independent HPLC runs by means of comparison with the internal standard C_{80}-polyprenyl-P (Fig. 2C) confirmed this observation, showing that every single Dol-P species was increased in ZA treated fibroblasts and that the Dol-P tended to

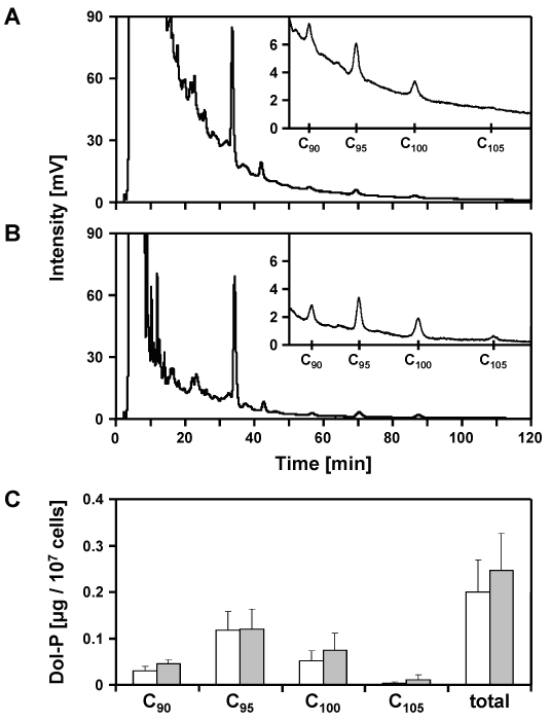

Figure 2: Analysis and quantification of Dol-P levels in mock and ZA treated control fibroblasts. **(A)** Control cells were grown in the presence of DMSO. Cellular Dol-P were prepared, labelled with the fluorophore 9-anthryldiazomethane and subsequently separated by HPLC. The enlarged section in the fluorescent profile highlights the separation of the particular Dol-P species. The elution times of mammalian Dol-P standards ranging from C_{90}- to C_{105}-Dol-P are indicated below the section. The internal C_{80}-polyprenol-P standard utilized for quantification eluted at a retention time of 33.5 min. **(B)** The separation of Dol-P from ZA treated control fibroblasts is presented in the enlarged section, and the respective Dol-P species were assigned like in **(A)**. Quantification was accomplished by comparison with the internal standard hexadecaprenyl-P eluting at 34.2 min. **(C)** The Dol-P levels from four independent HPLC runs were calculated and normalized to 10^7 fibroblasts. White bar represent Dol-P originating from mock treated and gray bars Dol-P from ZA treated cells. Error bars exhibit the standard derivations.

increased isoprene subunit numbers. The overall increase of the entire Dol-P pool was considerably lower in human fibroblasts than observed in rat livers (Keller, 1996), yet reaching a level of 123% compared to untreated cells. Hence, ZA has the ability to stimulate the biosynthetic formation of Dol-P in cultured human fibroblasts, featuring a preference for longer Dol-P.

In the case of DPM1 deficiency, mutations within the catalytic subunit of the Dol-P-Man synthase complex impede the formation of the sugar building block Dol-P-Man (Kim, et al., 2000). In our hands, the [^{3}H]-Dol-P-Man pool determined in untreated DPM1-CDG fibroblast was reduced by half compared to control cells (Fig. 3). Reconstitution to normal levels of the mono-saccharide donor would be of importance considering the dependent GPI-anchor formation and LLO biosynthesis. When DPM1-deficient fibroblasts were treated for 72 h with 100 µM ZA, the Dol-P-Man amount was replenished to 78% of wild-type levels. The immediate need for the Dol-linked glycosylation substrate might explain the incomplete restoration, and monitoring of large quantities of isotopic labelled short length LLO in the TLC lanes corresponding to DPM1-CDG deriving samples support this assumption (data not shown). However, ZA administration has no big influence on [^{3}H]-Dol-P-Man in healthy fibroblasts (Fig. 3), probably due to down-regulation of DPM activity (Banerjee, et al., 2005).

DPM1 deficiency leads to considerably reduced cell surface expression of GPI-anchored proteins, such as CD59, a component of the complement system (Imbach, et al., 2000b). As estimated, we could show that ZA also increases the expression of CD59 by improved disposability of the GPI progenitor Dol-P-Man. In fact, both control (Fig. 4A) and DPM1-CDG fibroblasts (Fig. 4B) presented a markedly increased CD59 cell surface expression

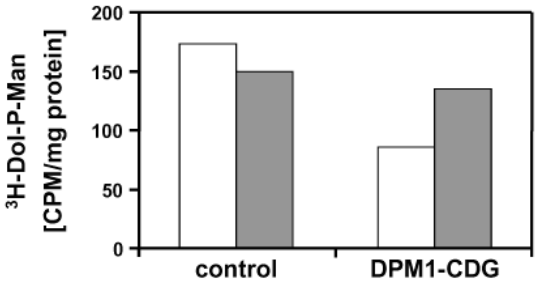

Figure 3: Determination of Dol-P-Man levels in mock and ZA treated fibroblasts. Dol-P-Man was metabolically labelled in control and DPM1-CDG fibroblasts by incorporation of [^{3}H]-Man and thereafter extracted. The purified lipids were separated by TLC and, on the basis of radiography, the regions corresponding to [^{3}H]-Dol-P-Man were scraped and quantified by liquid scintillation. Normalization of Dol-P-Man levels against the protein amounts of mock treated cells are shown as white bars, while the normalized values of ZA treated fibroblasts are shown as gray bars. The depicted graph is a representative of two independent experiments.

upon ZA treatment, as monitored by flow cytometry. When DPM1 deficient fibroblasts were treated for 72 h with 100 µM ZA, CD59 expression returned to normal levels like observed for untreated control cells (Fig. 4C), therewith fully restoring the healthy situation. Monitoring of CD59 levels turned out to serve as manageable and exactly quantifiable assay to determine optimal time and dosage applications of ZA. A time scale experiment (Fig. 4C) showed that ZA completely commences its effect after an incu-bation time of 72 h and longer administration raises CD59 expression only minimal. On this account, all experiments were performed after statin administration of three days. Moreover, control and patient fibroblasts were cultivated in the presence of increasing amounts of ZA, and CD59 cell surface expression was monitored by flow cytometry to define optimal ZA dosage. The dosage dependent increase of CD59 levels reached a maximum at the end point concentration of 100 µM ZA, which was set as standard dose.

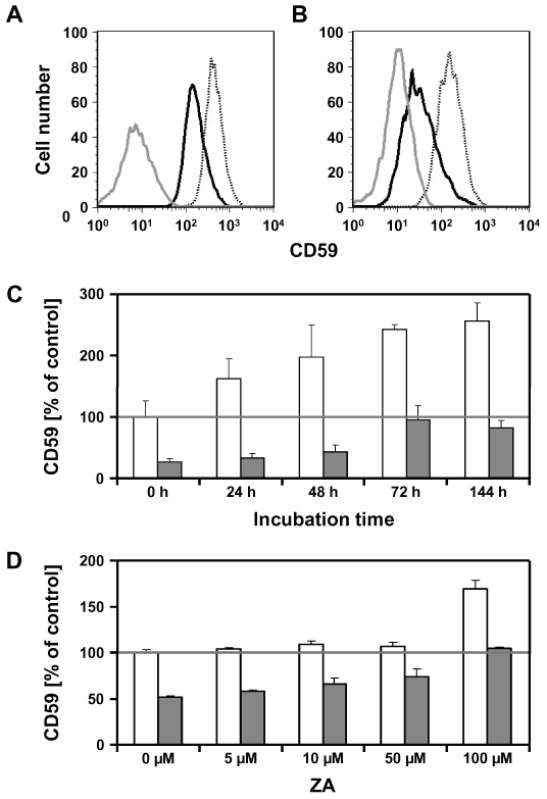

Figure 4: Cell surface expression of GPI-anchored CD59. **(A)** Control fibroblasts were stained with a FITC conjugated α-human CD59 antibody and analysed by flow cytometry. The fluorescent histogram shows unstained cells as gray solid line, mock treated cells as black solid line and ZA treated cells as black dotted line. **(B)** CD59 expression on DPM1-CDG patient fibroblasts was monitored by flow cytometry. Experimental set up and specimen allocations were like for control cells in **(A)**. **(C)** Control (white bars) and DPM1-CDG fibroblasts (gray bars) were incubated with 100 µM ZA for varying time periods and the detected CD59 expressions were normalized to CD59 levels of untreated control fibroblasts. Each bar represents the average of three independent experiments and the error bars exhibit the standard derivations. **(D)** Different concentrations of ZA were administered to control (white bars) or DPM1-CDG (gray bars) patient fibroblasts for 72 h. As in **(C)**, the measured CD59 expressions were normalized to untreated control cells. The experiments were performed in triplicates and the standard derivations are given by the error bars.

Reduced levels of GPI-anchored proteins constitute a molecular aspect of individual CDG, however, each biosynthetic CDG exhibits diminished biosynthesis of the Dol-linked oligosaccharide $GlcNAc_2Man_9Glc_3$. Glycosyltransferase defects lead to accumulations of incomplete LLO intermediates, which are transferred to some extent to target proteins by the oligosaccharyltransferase enzyme complex (Grubenmann, et al., 2002; Chantret, et al., 2003). Accordingly, insufficient supply with complete LLO precursor raises under- and miss-occupancy of N-glycosylation sites in target glycoproteins (Haeuptle and Hennet, 2009). Certainly, the accumulated LLO intermediates, which served as useful attribute for the identification of proximate defective enzymatic steps (Grubenmann, et al., 2002; Chantret, et al., 2003; Kranz, et al., 2004), were always accompanied by full length LLO precursor. A potential therapeutic agent would therefore aim to change the ratio of pathogenic intermediate to complete LLO. The HPLC profiles of DPM1-deficient patients depicted the accumulation of the Dol-linked intermediates $GlcNAc_2Man_5$ to $GlcNAc_2Man_8$ (Fig. 5C) (Imbach, et al., 2000b; Kim, et al., 2000) induced by reduced disposability of Dol-P-Man, the donor substrate for the mannosyltransferases catalyzing the addition of the fifth to the ninth Man. However, the amount of the major intermediate Dol-PP-$GlcNAc_2Man_5$ could be significantly reduced upon treatment of DPM1-CDG fibroblasts with ZA and simultaneously, the portion of full length LLO $GlcNAc_2Man_9Glc_3$ could be augmented (Fig. 5D). By comparison, treatment of control fibroblasts with ZA had no influence on the LLO profile (Fig. 5A and 5B). Thus, ZA has also the ability to induce the formation of full length Dol-linked N-glycosylation precursor as shown in DPM1-CDG fibroblasts.

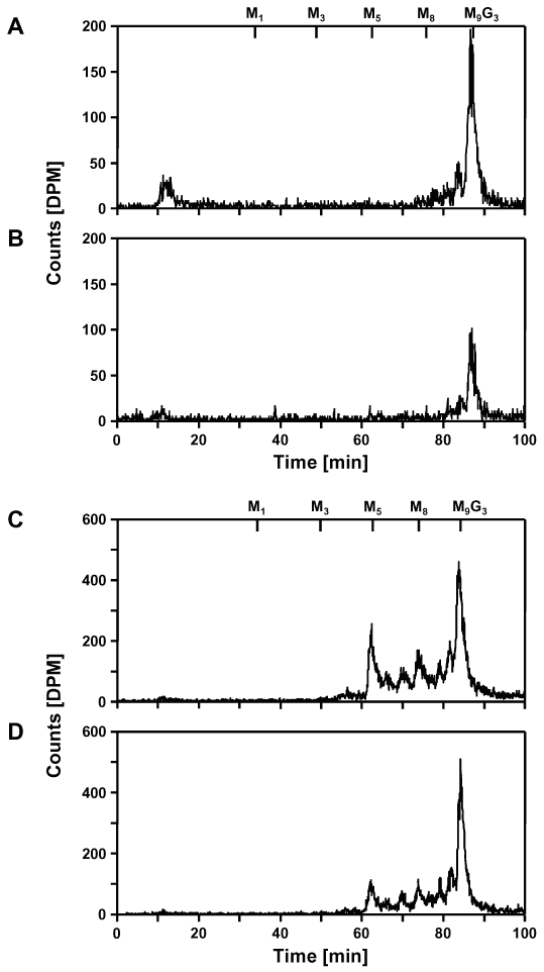

Figure 5: Analysing the effect of ZA on LLO. Fibroblasts derived from a healthy control **(A** and **B)** or a DPM1-CDG patient **(C** and **D)** were treated for 72 h either with 100 µM ZA **(B** and **D)** or DMSO as control **(A** and **C)**. Thereafter, the cells were metabolically labelled with [^{3}H]-Man and the LLO were extracted and hydrolysed by mild acid treatment. The released oligosaccharides were purified and separated by HPLC. The retention time of standard yeast oligosaccharides ranging from $GlcNAc_2Man_1$ (M_1) to $GlcNAc_2Man_9Glc_3$ (M_9G_3) are indicated at the top of the profiles.

The ZA induced amendment of LLO should also be reflected on the level of protein attached glycans. In the case of DPM1 deficiency, the accumulated LLO $GlcNAc_2Man_5$ to $GlcNAc_2Man_8$ were thoroughly transferred to glycoproteins (Fig. 6C). After trimming of the NLO by endoplasmic reticulum resident mannosidases, the HPLC profile exhibits the presence of $GlcNAc_2Man_4$ to $GlcNAc_2Man_7$, and the wild-type oligosaccharides $GlcNAc_2$-Man_8, $GlcNAc_2Man_9$ and $GlcNAc_2Man_9$-Glc_1 were explicitly under-represented (Fig. 6A). While ZA treatment did not affect the NLO profile of control fibroblasts (Fig. 6B), it significantly reduced the occurrence of abnormal N-linked glycans $GlcNAc_2Man_4$ to $GlcNAc_2Man_7$ and in return conveyed the formation of wild-type NLO (Fig. 6D). The improved ratio of normally N-linked glycans to incompletely glycosylated NLO demonstrates that ZA has eventually a favourable effect on protein N-glycosylation in CDG fibroblasts.

The ZA induced stimulation of Dol-P biosynthesis yielded in enhanced formation of Dol-P-Man, most probably due to elevated substrate availability. Dol-P-Man serves as donor substrate for GPI anchor formation as well as LLO biosynthesis, and accordingly, both parameters are elevated upon ZA treatment. The consequential increase of complete LLO precursor facilitates a normalization of protein N-glycosylation, which might be taken as conclusive evidence for the potential of ZA as CDG treating agent.

Discussion

Successful treatment of biosynthetic CDG was restricted so far to orally administration of Man to Man phosphate isomerase deficient (MPI-CDG) patients (Niehues, et al., 1998; Westphal, et al., 2001b). The applied Man could be phosphorylated by hexokinase and allows in doing so the functional bypass of the defective isomerization of fructose-6-P to Man-6-P. In the group of processing CDG, a deficiency of the Golgi GDP-Fuc transporter (SLC35C1-CDG or leukocyte adhesion deficiency type II) (Lübke, et al., 2001) could also be treated by simple supplementation of a monosaccharide. Oral Fuc administration induced

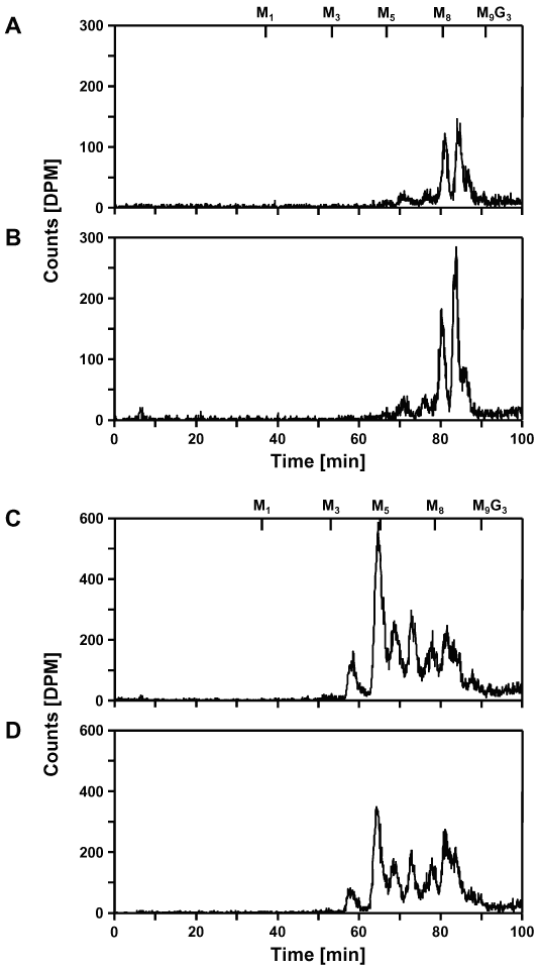

Figure 6: Protein N-glycosylation in ZA treated fibroblasts. NLO from control **(A** and **B)** and DPM1-CDG patient fibroblasts **(C** and **D)** were separated by HPLC. The protein linked oligosaccharides were prepared by N-glycosidase F release from metabolically labelled cells previously treated for 72 h either with 100 µM ZA **(B** and **D)** or DMSO as control **(A** and **C)**. The retention times of $GlcNAc_2Man_1$ (M_1) to $GlcNAc_2Man_9Glc_3$ (M_9G_3) are marked at the top of the profiles.

the expression of fucosylated glycoproteins and within short terms, the clinical symptoms of a SLC35C1-CDG patient could be relieved (Marquardt, et al., 1999). Moreover, a few elementary attempts have been directed to treat phosphomannomutase 2 deficiency (PMM2-CDG), which forms by far the largest CDG subtype (Freeze, 2009). Mutations in the *PMM2* gene lead to disturbed conversion of Man-6-P to Man-1-P, which acts as progenitor of GDP-Man (Matthijs, et al., 1997). However, Man-1-P is neither able to diffuse through biological membranes nor exists a transport system and it must therefore be chemically modified prior to direct administration to overcome this obstacle (Rutschow, et al., 2002; Eklund, et al., 2005a; Hardre, et al., 2007). Membrane permeable Man-1-P analogues were successful in restoring LLO biosynthesis in CDG patient cells, but such prodrugs were toxic, very unstable and had to be applied in high concentrations (Eklund, et al., 2005a).

Our approach to manipulate the Dol-P biosynthesis by inhibition of the squalene synthase offers an option for inherited biosynthetic N-glycosylation defects featuring residual enzymatic activity. Conveniently, ZA depicts an improved tolerance in animal experiments (Baxter, et al., 1992; Bergstrom, et al., 1993) compared to classical statins, which are assumed to generate a part of their numerous adverse effects (Silva, et al., 2007) by inhibition of the diverging pathways such as isoprenoid synthesis (Fig. 1A). Either way, we could demonstrate that the ZA induced formation of the N-glycosylation substrate Dol-P (Fig. 2) was sufficient to improve cellular levels of the monosaccharide donor Dol-P-Man (Fig. 3) and the GPI-anchored protein CD59 (Fig. 4). In addition, the ZA increased substrate availability lead to correction of pathologically altered LLO (Fig. 5) and NLO (Fig. 6) patterns in DPM1-CDG patient fibroblasts.

This study has been restricted to the use of DPM1-deficient fibroblasts as CDG model due to its numerous cellular symptoms determined by incomplete N-glycosylation and GPI-anchor formation. For instance, PMM2-deficient patients form although the largest group of CDG patients

(Haeuptle and Hennet, 2009), but they do not feature any accumulation of LLO intermediates and consequently no transfer of immature glycans to glycoproteins. However, the fact that the glycosyltransferases catalysing the attach-ment of the last four Man and three Glc of the LLO precursor depend on Dol-linked monosaccharide precursors highlights the broadness of a possible application range (Kornfeld and Kornfeld, 1985). Above all, we could observe that diminished expression levels of GPI-anchored CD59 in MPDU1-CDG (Schenk, et al., 2001b) and unassigned CDG patient derived fibroblasts could be restored upon ZA treatment (data not shown). The individual impact of ZA might not be enormous, but taking in account that mutated genes in CDG exhibit residual enzymatic activity, this might already be sufficient to substantially approach non-pathological glycosylation levels.

Over the last decade, complete gene knock out mouse models for PMM2-, MPI- and DPAGT1-CDG have been generated (Marek, et al., 1999; DeRossi, et al., 2006; Thiel, et al., 2006). Obviously, disruption of those genes is not compatible with life and embryos homozygous for the null alleles were recovered between days 2.5 - 11.5 post fertilization. It would be interesting to investigate whether the embryonic lethality of the homozygotes could be lagged to a later stage of development or even to nativity by treating pregnant heterozygous mice with ZA according to Keller (Keller, 1996). In addition, the generation of viable CDG mouse models, for instance by engrafting hypomorphic alleles, would allow detailed analysis of organ specific effects of ZA in the context of inherited N-glycosylation deficiencies. This seems to be of peculiar interest, considering that Keller reported an enormous effect of ZA on Dol and Dol-P pools in rat livers, but not in other organs such as brain, kidney, intestine or testis (Keller, 1996). Subcutaneously administered ZA might preferentially taken up by mammalian livers via a specific hepatic transport mechanism, which was likewise proposed in other studies (Baxter, et al., 1992; Bergstrom, et al., 1993). Combined with the knowledge of frequent derogation of liver function in CDG patients (Leroy, 2006), the proposed hepatic transport system for ZA suggests to turn the attention of prospective research to the metabolic organ of the opted model organism. Moreover, potential adverse effects of ZA administration to mammalian CDG organisms could likewise be monitored.

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General Discussion

Why CDG research?

While working on this thesis I have been repeatedly confronted with the question of spending so much attention to rare inherited diseases. The general opinion understands that biological research should be focused on the most "important" health problems, such as cancer, infectious diseases or cardiovascular disorders. But in thinking so, one does not take in account that the pharmaceutical industry dedicates much attention to these themes. Although academic research is accountable too, it has the advantage to be less restricted in choosing the topics, since it is less dominated by economic motives. However, current research and development is so networked and interdisciplinary that the findings of biomedicine might be beneficial to many other fields. Thus, I would like to elaborate on the basis of CDG, why research on a "remote" topic can be considered reasonable and interesting.

The recent description of five patients with mutations in the *RFT1* gene (Haeuptle and Hennet, 2009) demonstrated the importance of CDG research and especially the necessity of continuous screening for novel defects. The erroneous believe that the features of a single subtype of a rather rare disease might only concern researchers of this field and the affected patients' families can be refuted by several arguments regarding clinical, biological and biophysical aspects. The exact descriptions of the clinical and biochemical manifestations of such disorders are of great interest for pediatricians all over the world, which have to deal with patients presenting with multi systemic symptoms. Just as important is the knowledge about biological processes, gained by accurate observation of the impact of a single defective enzymatic activity on an entire organism. Researchers argue for instance intensely about the exact function of the RFT1 protein. While Helenius and coworkers assigned the encoded product of the *Saccharomyces cerevisiae* gene *Rft1* in genetic complementation assays as flippase responsible for the translocation of the Dol-PP-$GlcNAc_2Man_5$ intermediate across the ER membrane (Helenius, et al., 2002), members of the Menon lab did not detect this activity by *in vitro* assays (Frank, et al., 2008; Sanyal, et al., 2008). Accordingly, they postulated for RFT1 a different role as Dol-linked oligosaccharide chaperone, controlling the lateral distribution of LLO on the cytosol facing side of the ER. If our predicted RFT1 protein model (Haeuptle and Hennet, 2009) depicts the correct orientation with respect to the ER membrane, the five identified point mutations would locate to highly conserved loops protruding to the ER lumen. In combination with the observation that RFT1 deficiency led to disturbed LLO intermediate translocation, this would contradict a function of RFT1 as cytosolically acting LLO chaperone.

Besides contributing to the assignment of a controversial function to the RFT1 protein, an N-glycosylation defect accounted already earlier for the identification of the biological function of a

particular protein. Mutations within the *MPDU1* gene encoding the human Man-P-Dol utilization defect 1 protein, which is orthologous to the Chinese hamster protein Lec35 (Anand, et al., 2001) led to MPDU1-CDG. The patients exhibited the accumulation of Dol-linked $GlcNAc_2Man_5$ and $GlcNAc_2Man_9$ in HPLC profiles, a clear indication that MPDU1 is involved in the allocation of the lipid-linked monosaccharide donors Dol-P-Man and -Glc at the ER membrane (Kranz, et al., 2001; Schenk, et al., 2001b). Hence, protein defects cause not only diseases, but also contribute to understand the functional process, which might be helpful when searching for treating possibilities.

Likewise, genetic disorders served often as a valuable tool to identify and clone the mutated genes. Correspondingly, the *PMM2* gene was first cloned and studied in the context of CDG (Matthijs, et al., 1997), and complement cloning lead to the identification of the *SLC35C1* gene encoding the Golgi GDP-fucose transporter and, when mutated, causing leukocyte adhesion deficiency type II or SLC35C1-CDG (Lübke, et al., 2001). Moreover, it is expected that further enzymatic activities will be definitely linked to so far undefined genes upon characterization of the corresponding glycosylation disorder. Thus, CDG can be used as multi-functional instruments for glycobiologists uncovering the biological significance of glycosylation pathways.

Inherited versus acquired glycosylation deficiency

Multisystemic presenting disorders, such as CDG, are clinically difficult to diagnose and might have to some extent very diverse origins. Genetic alterations of completely different pathways could lead to a similar phenotype, however, intoxications could have the same consequences. Strikingly, the symptoms of the inherited disorder CDG are comparable with the manifestations of the acquired disease alcohol abuse at the molecular and cellular levels. Thus, the IEF profile of serum transferrin in CDG patients and chronic alcohol abusers cannot be discriminated, since both present similar under-glycosylated transferrin (Henry, et al., 1999). Similarly, the long term consequences of alcoholism resemble the clinical symptoms of CDG (Leroy, 2006). Chronic alcohol abuse was found to damage nearly every organ, whereas prominent are cardiovascular symptoms, liver disease, damages of the central and peripheral nervous system, pancreatitis and cancer (Testino, 2008). In contrast to CDG, not all long term alcohol abuse symptoms are generated by deficient protein glycosylation. Nevertheless, under-glycosylated proteins might contribute at least to a part of them. In any case, both fields of research, combating either the inherited or the acquired form of protein under-glycosylation, could profit from the other. The IEF of transferrin, allowing the diagnosis of both CDG and chronic alcohol abuse, sets a good example that the variable methods established for the identification and characterization of CDG might contribute to understand the molecular reasons of the fatal outcome of alcohol abuse. In return, long term administration of alcohol to mammalian organisms could simulate a state of

protein under-glycosylation (Cottalasso, et al., 1996) and therewith serve as commonly applicable model for deficient protein glycosylation.

In Switzerland, estimated 4% of the population suffer from alcoholism (www.swissworld.org). As sad those individual fates might be, they nevertheless contribute virtually to the significance of CDG research. Knowledge about imperfect glycosylation gained by CDG researchers might be useful to understand and hopefully improve the state of health of active or reformed long-time alcohol abusers. And all of a sudden, such a "remote" research field has been linked directly to one of the biggest global health and social problems.

Prospective targets in CDG research

At the end of my thesis I dare to claim to be competent enough to predict in which direction CDG research should and will be oriented. Within the biosynthetic LLO pathway, being the main topic of my doctoral work, most of the involved genes have been associated with a form of CDG. So far unaffected genes like *DPM2*, *ALG5*, *ALG13/14* or *ALG11* are assumed to be the next ones to be associated with novel forms of CDG. A special case forms the OST complex, which is responsible for the transfer of mature LLO to target glycoproteins. Its oligomeric organization complicates the screening for mutations and the proof of pathogenicity immensely. Furthermore, mutations in the OST subunit encoding genes *N33/TUSC3* and *IAP* seem not to have any influence on transferrin N-glycosylation (Molinari, et al., 2008). For this reason, potential OST deficient patients are assumed to escape the classical CDG diagnosis method and are therefore hardly detected by the current screening methods.

The recent discovery of Dol-kinase deficiency as causing CDG (Kranz, et al., 2007b) opened new perspectives with regard to the biosynthetic defects. Hence, non assigned CDG patients displaying low rates of LLO biosynthesis could likewise suffer from incomplete supply with the N-glycosylation membrane anchor Dol-P. Our established Dol-P analysis method could serve as adequate tool for screening of remaining defects in Dol-P biosynthesis. Hot candidates constitute the genes encoding the *cis*-prenyltransferase, the Dol-reductase and the polyisoprene mono- and pyrophosphate phosphatases (Schenk, et al., 2001a).

The group of N-glycan processing defects was originally populated by defective glycosyltransferases (Tan, et al., 1996; Hansske, et al., 2002), a glycosidase (De Praeter, et al., 2000) and transporters of monosaccharide donors (Lübke, et al., 2001; Martinez-Duncker, et al., 2005), all required for protein N-linked glycan modifications. Though, this subgroup was expanded shortly by several new members impairing the transport of N-glycoproteins along the secretory pathway (Wu, et al., 2004; Foulquier, 2008; Kornak, et al., 2008). Simultaneously, the range of candidate genes was raised several fold, given that dysfunction of any protein of the

secretory pathway involved in the maturation and transport of glycoproteins (Helenius and Aebi, 2001) could lead to CDG.

Despite intensive research for over two decades, only two types of CDG can effectively be treated. Oral supplementation of Man and fucose to Man phosphate isomerase (MPI-CDG) and GDP-fucose transporter (SLC35C1-CDG) deficient patients, respectively, restored normal glycosylation and health condition (Niehues, et al., 1998; Marquardt, et al., 1999). The unfavorable fate of more than 1000 CDG patients (Haeuptle and Hennet, 2009) calls urgently for the development of therapeutical approaches. Since gene therapy is not a valuable and practicable option, we have tried to up-regulate glycosylation pharmacologically. Increased formation of the N-glycosylation substrate Dol-P by inhibition of the squalene synthase with ZA was substantially able to correct the various manifestations of CDG in patient fibroblasts. Though, the cleft between cell culture experiments and the treatment of human patients is immense. Accordingly, administration of ZA to an appropriate mouse model is considered as next consequential step to close the huge gap. Hopefully, it will contribute to the alleviation of the various and severe symptoms of CDG, which composes, in my eyes, one of the major motive forces of medical research.

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